Chemical interactions between aquatic microorganisms

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Chemical Interactions

Between Aquatic Microorganisms

Habilitationsschrift

vorgelegt von

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Parts of this thesis have been published or patented.

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Abbreviations

A	Alanine
Adda	(*2S,3S,8S,9S*)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
Ahp	3-Amino-6-hydroxy-2-piperidone
Ala	Alanine
allo-Ile	*allo*-Isoleucine
Allyl-	H_2C=CH-CH_2-R; substituent, where R is the connection to the rest of the molecule
Arg	Arginine
Asp	Aspartic acid
Bcc	*Burkholderia cepacia* complex
Benzyl-	$C_6H_5CH_2$-R, substituent, where R is the connection to the rest of the molecule
C	Cysteine
C8-HSL	N-Octanoylhomoserine lactone
CARD-FISH	Catalyzed reporter deposition-fluorescence *in situ* hybridization
CP	Cyanopeptoline
Cys	Cysteine
DCP	Dichlorophenole
Dha	2-Aminoacrylic acid; Dehydroalanine
Dhb	2-Amino-2-butenoic acid; Dehydrobutyrine
DifMUP	Difluoromethylumbelliferyl phosphate
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig; Germany
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
ESI-FTICR-MS	High-resolution electrospray Fourier transform ion cyclotron resonance mass spectrometry
F	Phenylalanine
G	Glycine
GC-MS	Gas chromatography-mass spectrometry
Gln	Glutamine
Glu	Glutamic acid
Hil	Homoisoleucine
Hmp	3-Hydroxy-4-methylproline
HPLC	High pressure-liquid chromatography
Hse	Homoserine
HSL	*N*-Acyl homoserine lactone
Hty	Homotyrosine
I	Isoleucine
IC	Inhibitory concentration
Ile	Isoleucine
i.p.	Intraperitoneal
L	Leucine
LC	Lethal concentration

LD	Lethal dose
Leu	Leucine
Lys	Lysine
M	Methionine
MAR	Microautoradiography
MC	Microcystin
Mdha	*N*-Methyldehydroalanine
Mdhb	Methyldehydrobutyrine
MeAsp	Methylaspartic acid
MeOH	Methanol
MeOzn	Methyloxazoline
MeSer	Methylserine
Met	Methionine
MeTyr	*N*-Methyltyrosine
MIC	Minimal inhibitory concentration
MPC	Minimal phytotoxic concentration
MTBSTFA	*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide
NMR	Nuclear magnetic resonance
PCC	Pasteur Culture Collection of Cyanobacteria; Paris; France
Phe	Phenylalanine
PP1	Protein phosphatase(s) 1
PP2A	Protein phosphatase(s) 2A
PRN	Pyrrolnitrin
R	Arginine
S	Serine
SAG	Sammlung von Algenkulturen; Göttingen; Germany
SAR	Structure-Activity-Relationship
Ser	Serine
T	Threonine
Thr	Threonine
TLC	Thin-layer chromatography
Tzl	Thiazole
Tzn	Thiazoline
Tyr	Tyrosine
V	Valine
Val	Valine
Y	Tyrosine

1 Introduction

1 Introduction

Section 1.1 Aquatic Chemical Ecology

'Chemical Ecology' describes in general the study of **bioactive compounds** that mediate interactions and the information transfer within and between living organisms. In contrast to the study of "Natural Products" the main focus is on the **ecological understanding** of the origin, the presence, and the function of such compounds. Although main work done so far has considered mainly terrestrial environments, "Aquatic Chemical Ecology" is a rapidly growing discipline that became more and more important during the last few decades (Hay, 2002). Chemical defences of prey organisms, chemically mediated foraging, habitat selection, host-symbiont interactions, mate and swarm recognition, and quorum sensing as well as pheromone sensing have been conducted so far in aquatic systems (Hay, 2002, Hay & Kubanek, 2002). The close collaboration between biologists and chemists has helped to work out new insight into these interactions in nature (Hay, 2002). The current terminology to classify bioactive compounds in general is quite heterogeneous as every definition was made in a specific context (Willis, 2007). In the next two sections the determination of **bioactive compounds** throughout this thesis will be explained. Generally, all definitions used in the text are made based on their effect, regardless their structure.

Section 1.2 Pheromones and Allelochemicals

Pheromones and allelochemicals are organic compounds that transmit chemical messages. Both classes belong to subgroups of the so called secondary metabolites that are believed not required for metabolism such as growth, reproduction, and development (Whittaker & Feeny, 1971). The term "pheromone" was introduced already in the middle of the last century based on the Greek words φέρειν (to transport) and όρμον (to stimulate) (Karlson & Lüscher, 1959). By definition **pheromones** are compounds that are released into the surrounding, and which cause specific reactions in members of the same species (***intra*specific**) (Karlson & Butenandt, 1959, Karlson & Lüscher, 1959). These substances are well studied, and are most often grouped according to their characteristics. Very well known are the so called sex pheromones, mainly known from insects that are released to attract mates, and can be recognized even from as far away as several kilometres (Raina*, et al.*, 1989). By contrast, **allelochemicals** (based on the Greek word αλληλος 'another') are substances, which influence growth, survival and reproduction of organisms of different species (***inter*specific**) (Whittaker & Feeny, 1971, Whittaker, 1975). Allelochemicals might be also released into the environment, but are stored insight the cells/organisms.

Section 1.3 Classification of Allelochemicals based on their Effect:

During the last decade, research on allelochemicals in aquatic environments has increased markedly, which articulates not only the importance, but also the increasing interest to gain more knowledge about these bioactive compounds. In this thesis the allelochemicals are classified as follows:

A) Toxins

In general, toxins are poisonous products of organisms, harmful to organisms of another species. Toxins are known to be produced by all possible kind of organisms, such as microorganisms, algae, plants and animals. These compounds have predominantly two functions: they are used by predators for hunting purpose or as a defence mechanism to avoid predation. In this thesis toxins are considered to only affect heterotrophic multicellular eutrophic organisms, such as (aquatic) invertebrates and mammals (including humans), but not bacteria, protists, algae, fungi, and plants.

B) Allelopathically active compounds

In contrast, allelopathy covers interactions between primary producers and microorganisms or among primary producers themselves (Molisch, 1937). Allelopathically active compounds are produced by plants, microorganisms, or fungi (Willis, 1985), and mostly released into the environment to inhibit the growth and the reproduction of other microorganisms.

C) Infochemicals

Infochemicals play an important role in shaping the community structure of individuals either from the same or from other trophic levels (Schoonhoven, 1968, Nordlund*, et al.*, 1981). In general, chemoreceptors of recipient organisms perceive and process the information that is mediated by infochemicals to either accept an operation (e.g. movement to a higher concentration of the targeted compound) or to reject an action in progress (e.g. stop the ingestion of food organisms) (Dethier, 1947, Schoonhoven, 1968, Miller & Strickler, 1984, Renwick & Radke, 1987). Infochemicals are prepared to gain an advantage by the producing organism. A special case of infochemicals are the so called kairomones, that are released into the environment and are in most cases and advantage only for the recipient, but not for the producer (Brown Jr.*, et al.*, 1970).

Section 1.4 "Chemical interactions between Aquatic Microorganisms"

In my work, I will follow the here proposed classification of allelochemicals. During the last years I worked with all three types of compounds albeit different key aspects. Therefore, I subdivided this thesis into three parts:

Chapter 2) **Cyanobacterial Peptide Toxins**. Main focus of this work was the detection, the characterisation, and the structure elucidation of several typical peptide toxins of aquatic cyanobacteria with analytical methods, the characterisation of their toxicity, and the search for their possible mode of action. Additionally, metabolic characterisation of cryptic ecotypes in the toxic cyanobacterium *Planktothrix* spp. are currently examined (Outlook).

Chapter 3) **Allelopathy - Growth Inhibitors/Promoters**. In this part all work was combined that includes microbial growth inhibition albeit different approaches used in the studies. Structure-activity relationship (SAR) studies were used to characterize the correlation between the three-dimensional structure of the target molecule and its biological activity, or rather the chemical groups responsible for the observed effect (work done on nostocarboline and anachelin). In two other studies, the main focus was the growth inhibition effects by different type of extracts (from protists or *Planktothrix*) on typical freshwater bacteria. Finally, analytical and molecular work on the antifungal agent pyrrolnitrin, produced in members of the genus *Burkholderia*, was included into this chapter.

Chapter 4) **Infochemicals - Kairomones**. This work focussed on the establishment of a bioassay to assess the bacterial aggregate formation in the presence of bacterivorous flagellates. It could be shown that this morphological change was not only affected by growth state, but also by the presence of conspecific chemical cues. Therefore, this bioassay is now used for bioassay-guided fractionation of putative kairomone-containing solutions and to assess experimental bacterial evolution in the presence of grazers (Outlook).

Information: Bold marked references in the text refer to own publications, and work that was done in collaboration with other research groups.

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2 Cyanobacterial Peptide Toxins

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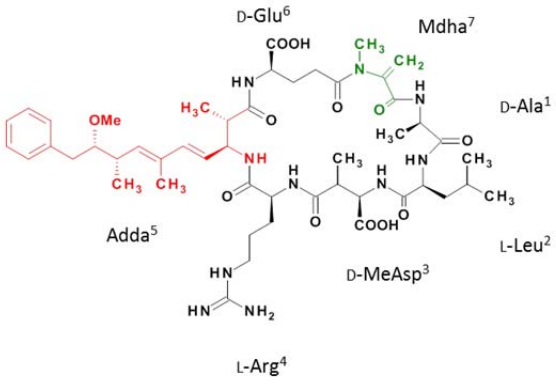
Cyanobacteria have gained worldwide importance as their intense growth is favoured by anthropogenically induced nutrient enrichment such as untreated and insufficiently treated wastewaters, fish farming, or the run-off of over-fertilized farmland (Smith, 2003). Under high nutrient load (particularly low N/P ratio), cyanobacteria tend to become the dominant primary producers in aquatic ecosystems (Paerl, 1996). Mass occurrences of cyanobacteria are frequently observed in mesotrophic and eutrophic lakes (Sivonen & Jones, 1999). The genera *Microcystis* and *Planktothrix* are the most common phytoplankton organisms that develop high densities in freshwater lakes, either at the surface (*Microcystis*) or in deeper water layers (*Planktothrix*) (Chorus & Bartram, 1999). However, cyanobacterial blooms do not only represent aesthetic problems particularly when the cyanobacterial cells lyse and release the intensively blue or red coloured phycobilins, or strongly odorous compounds. These blooms can also cause severe problems in reservoirs and lakes used as drinking water supplies, recreation areas, and watering places for livestock due to the production of toxic metabolites that can be harmful to animals, cause skin irritations of swimmers and may cause health effects via aerosols (Chorus & Bartram, 1999). Raw water taken from lakes and rivers dominated by cyanobacteria have to be carefully treated to avoid hazardous effects on the consumers. The presence of secondary metabolites in bloom-forming cyanobacteria is a widespread and common phenomenon, either formed by nonribosomal peptide synthetases or through modifications of ribosomally synthesized pre-peptides. In recent years substantial progress has been made in the identification of harmful and **toxic oligopeptides** from the genera *Planktothrix* and *Microcystis*. Most of the hitherto described compounds can be assigned to distinct chemical classes (Welker & von Döhren, 2006). Historically, research has primarily focused on **microcystins** and their potential to inhibit protein phosphatases (Zurawell*, et al.*, 2005). So far, considerably less attention has been paid to the biological function and ecological effects of **cyanopeptolins** and **cyclamides**, two common oligopeptide classes produced by planktonic cyanobacteria (Welker & von Döhren, 2006), although oligopeptides from both groups showed acute toxicity for aquatic organisms comparable to the one of microcystins **(Blom*, et al.*, 2001, Blom*, et al.*, 2003, Portmann*, et al.*, 2008a, Portmann*, et al.*, 2008b, Gademann*, et al.*, 2010)**. While cyanopeptolins are known as potent inhibitors of trypsin-like proteases **(Gademann*, et al.*, 2010)**, the possible targets of cyclamides are presently unknown.

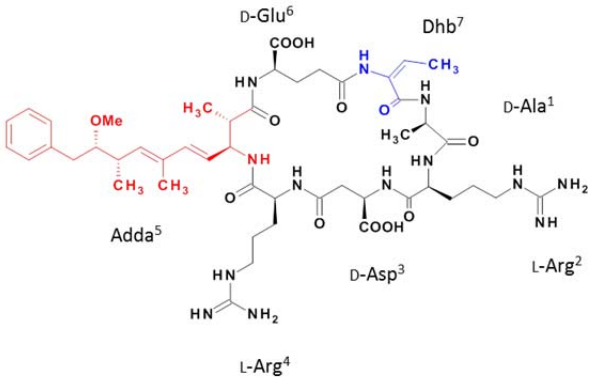
Section 2.1 General structure of microcystins

Microcystins (MCs) are cyclic heptapeptides that consist of the common structure *cyclo*-(D-Ala^{1}-L-X^{2}-D-MeAsp^{3}-L-Z^{4}-Adda^{5}-D-Glu^{6}-Mdha^{7}), where the superscript numbers indicate the position of the amino acid within the molecule. So far about 80 different congeners of microcystins have been described in the literature, but only a few structure elucidations have been confirmed by Nuclear Magnetic Resonance (NMR) studies (Welker &

von Döhren, 2006). Structural diversification is a result of amino acid substitutions in different positions of the molecule. Thereby, the letters X and Z in the general formula represent the most variable positions and are important for the microcystin nomenclature, as the identity of these amino acids is indicated using their one letter code. Thus, MC-LR **(1)** refers to a microcystin with leucine (Leu; L) and arginine (Arg; R) at the variable positions 2 and 4, respectively. Very few changes affect the positions 3 and 7. For position 3 only D-aspartic acid (D-Asp) or D-methylaspartic acid (D-MeAsp) have been found, whereas mainly *N*-methyldehydroalanine (Mdha; **(1)**, green coloured), dehydroalanine (Dha), or 2-amino-2-butenoic acid (Dhb; **(2)**, blue coloured) may occupy position 7 in the molecule. Very rarely, L-serine (L-Ser) or methylserin (L-MeSer) is incorporated at position 7. D-alanine at position 1 and the very unusual amino acid Adda ((*2S,3S,8S,9S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; **(1)** and **(2)**; red coloured) at position 5 are predominantly conserved in this class of compounds, and the glutamic acid (D-Glu) at position 6 is strictly conserved (Diehnelt*, et al.*, 2006). Changes compared to the common structure outlined above are denoted in brackets, e.g. [D-Asp^3,Dha^7]MC-HtyR bears a one homotyrosine (Hty) and one Arg (R) in positions 2 and 4, D-Asp instead of a D-MeAsp in position 3, and Dha instead of Mdha in position 7. During the last years the structure elucidation of three microcystins, isolated from different *P. rubescens* strains, were performed by applying various analytical techniques **(Blom*, et al.*, 2001, Christiansen*, et al.*, 2008)**.



MC-LR **(1)**

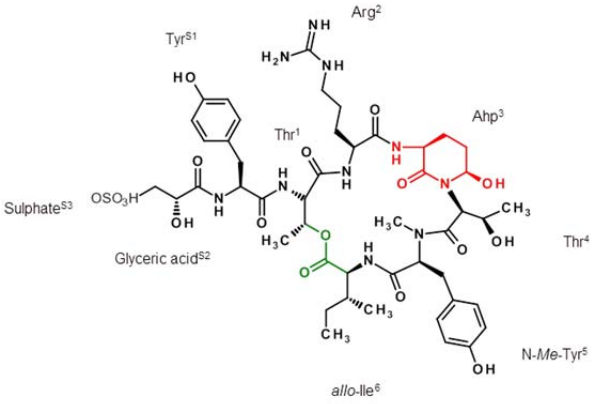


[D-Asp^3,(*E*)-Dhb^7]MC-RR **(2)**

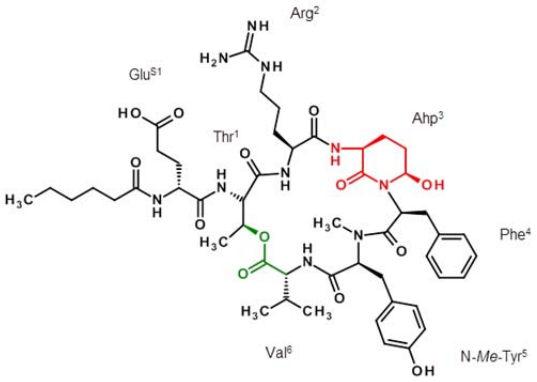
Section 2.2 General structure of cyanopeptolins

Cyanopeptolins belong to a large group of related cyclodepsipetides that feature the amino acid 3-amino-6-hydroxy-2-piperidone (Ahp) residue as a key structural unit (Welker & von Döhren, 2006). Thus, all of these compounds (such as ocscillapeptins as well as micropeptins, aeruginopeptins, nostopeptins and others) were merged to the major class of cyanopeptolins. Cyanopeptolins are generally widespread and have been isolated from

coccoid *Microcystis* as well as filamentous *Oscillatoria*, *Planktothrix*, *Nostoc*, and *Anabaena*. Concerning the structural diversity of these molecules the cyanopeptolins are by far the most abundant cyanobacterial peptides, and all can be considered inhibitors of proteases (Welker & von Döhren, 2006). Cyanopeptolins are hexa-, hepta-, or even octadepsipeptides that are not only characterized by Ahp in position 3 (**(3)** and **(4)**; red coloured), but also by the cyclization made by an ester bond of the β-hydroxy group of threonine (Thr) in position 1 (**(3)** and **(4)**; green coloured). Only in the nostopeptins this Thr is replaced by a 3-hydroxy-4-methylproline (Hmp) (Okino*, et al.*, 1997). All other positions in the ring system can be occupied by different amino acids. However, in position 5 only *N*-methylated aromatic



Oscillapeptin J **(3)**

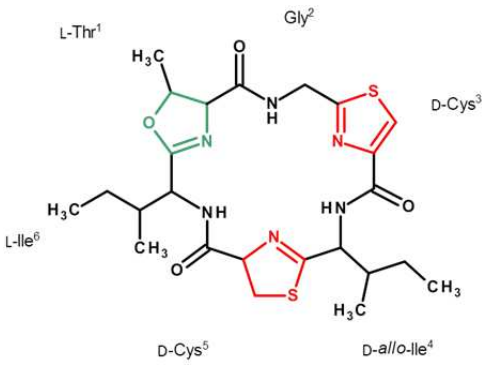


Cyanopeptolin 1020 **(4)**

amino acids have been reported so far (such as N-methylphenylalanine, N-methyltryptophan, or N-methyltyrosine) and in position 6 isoleucine (Ile) might be only replaced by valine (Val) or *allo*-isoleucine (*allo*-Ile). The highest structural diversity within the ring system is found for position 2, which can be occupied by various amino acids, e.g. Arg or Leu, or even homo-amino acids. There are two major types of side chains (Welker & von Döhren, 2006): on type consists of one to two amino acid(s) prolonged by an aliphatic fatty acid from variable length (such as found in cyanopeptolins 1020 **(4) (Gademann*, et al.*, 2010)**. The other type consists of a glyceric acid that can be either attached directly to the Thr in position 1 or via an amino acid side chain of variable length (Welker & von Döhren, 2006). Additionally, the glyceric acid might be partially methylated or esterified with sulphuric acid at the terminal position (Welker & von Döhren, 2006). A typical example of the second type of side chain is found in oscillapeptin J **(3) (Blom*, et al.*, 2003)**. During last years the structure elucidation of four cyanopeptolins, isolated from different cyanobacterial strains, was performed by applying various analytical techniques **(Blom*, et al.*, 2003, von Elert*, et al.*, 2005, Gademann*, et al.*, 2010)**.

Section 2.3 General structure of cyclamides

Another structurally intriguing class of cyanobacterial compounds is represented by cyclamides, a group of peptides that is in fact widely distributed but with a so far unknown biological function. Cyclamides are small cyclic peptides consisting of six amino acids. Typically, they consist of three unmodified amino acids that alternate in the molecule with amino acids (such as Thr or cysteine (Cys)) that are modified. These heterocyclized amino acids are then forming the characteristic thiazole/thiazoline (**(5)**; red coloured) and oxazole/oxazoline (**(5)**; green coloured) moieties in the cyclamides. Recently, four different cyclamides isolated from *M. aeruginosa* PCC 7806 were fully characterized **(Portmann*, et al.*, 2008a, Portmann*, et al.*, 2008b).** Additionally, a structure revision of microcyclamide 7806A was carried out **(Portmann*, et al.*, 2008b)**.



Aerucyclamide A **(5)**

Section 2.4 Toxicity of microcystins and their inhibition of protein phosphatases

Microcystins have been shown to be the causative agents for acute toxicity in aquatic animals and mammalian livestock (Dawson, 1998). Therefore, isolated and purified, as well as commercial available microcystin derivatives were tested for grazer toxicity against *Thamnocephalus platyurus* in 24-acute grazer toxicity assays **(Blom & Jüttner, 2005)**. Additionally, [D-Asp^3,(*E*)-Dhb^7]MC-RR was tested against several freshwater invertebrates, such as the rotifer *Brachionus calyciflorus*, insect larvae of *Chironomus riparius*, and *Daphnia* sp. and *Eudipatomus* sp., as representatives for filter-feeding and interception feeding crustaceans, respectively, and compared with the sensitive *T. platyurus* **(Blom*, et al.*, 2006)**. Biochemical studies have shown that the microcystins strongly inhibit protein phosphatases 1 (PP1) and protein phosphatases 2A (PP2A) of eukaryotic organisms (MacKintosh*, et al.*, 1990, Matsushima*, et al.*, 1990). The catalytic subunits of these enzymes belong to the most conserved proteins, and sequence identities of up to 90% were observed in organisms such as mammals, *Drosophila*, yeast and higher plants (Orgad*, et al.*, 1990, Sneddon*, et al.*, 1990). Inhibition of these serine/threonine phosphatases leads to the initiation of the apoptosis cascade and therefore, inevitably to cell death (Fladmark*, et al.*, 1999, Fladmark*, et al.*, 2002). The inhibitory activity of microcystins against PP1 and PP2A was repeatedly tested in enzyme inhibition assays, either by detection of radioactive labelled compounds (Xu*, et al.*, 2000), ELISA (Metcalf*, et al.*, 2001), or with colorimetric or fluorescent changes of the substrates used in these assays (An & Carmichael, 1994, Ward*, et al.*, 1997). Assays using fluorescence were found to be particularly sensitive. Originally, methylumbelliferyl phosphate was used (Mountfort*, et al.*, 1999), however, difluoromethylumbelliferyl

phosphate (DifMUP) was shown to be the even more sensitive and therefore the right substrate to test protein phosphatase inhibition (Fontal*, et al.*, 1999, Bouaïcha*, et al.*, 2002). Therefore, the inhibitory activity of nodularin and five different microcystin derivatives were tested in a protein phosphatase assay using DiFMUP as substrate for PP1 and PP2A **(Blom & Jüttner, 2005)**.

Section 2.5 Toxicity of cyanopeptolins, their potential to inhibit serine proteases, and adaptation of aquatic grazers to oscillapeptin J

Although several oscillapeptins have been isolated and described before (Shin*, et al.*, 1995, Itou*, et al.*, 1999, Fujii*, et al.*, 2000), toxicity against *T. platyurus* comparable with the one of microcystins was not known before oscillapeptin J was described **(Blom*, et al.*, 2006)**. Meanwhile, we could show acute toxicity against *T. platyurus* in the low micromolar range also for cyanopeptolin 1020 **(Gademann*, et al.*, 2010)**. Moreover, oscillapeptin J was tested against several freshwater invertebrates and compared with the toxicity of [D-Asp^3,(*E*)-Dhb^7]MC-RR **(Blom*, et al.*, 2006)**. In addition to aquatic grazers, oscillapeptin J was tested on mice as model organism for vertebrates **(Blom*, et al.*, 2006)**. Biochemical studies showed that all cyanopeptolins can be considered inhibitors of serine proteases. Although so far mainly mammalian enzymes have been tested in the context of pharmacological research, there is first evidence for a strong inhibition also of the digestive serine proteases of aquatic invertebrates (Baumann & Jüttner, 2006). Lastly, if oscillapeptin J is produced to provide defence against crustaceans, then adaptations of the grazer population may be measurable. Therefore, the sensitivity of *Eudiaptomus* sp. and *Daphina* sp. collected from two lakes, one with no occurrence of *P. rubescens*, the other with an annual massive bloom, was compared **(Blom*, et al.*, 2006)**.

Section 2.6 Bioactivity of cyclamides

Although various cyclamides structures have been described so far, there is not much known about their biological function and their ecological role. Some cyclamides have been found with multidrug reversing activities (Ogino*, et al.*, 1996), some exhibited moderate toxicity against sea urchin embryos (Admi*, et al.*, 1996), and others had strong antimalarial activity (Linington*, et al.*, 2007). Previously isolated from *Nostoc* strain 31, nostocyclamide exhibited values in the low micromolar range (LC_{50} value 13 µM) against different rotifers and crustaceans (Todorova & Jüttner, 1996). Thus, the toxicity of cyclamides against crustaceans is comparable with the one of microcystins **(Blom & Jüttner, 2005)**. Interestingly, heterotrophic bacteria such as *Alcaligenes eutrophus* and *Pseudomonas fluorescens* (both gram-negative) and the gram-positive *Arthrobacter globiformis* and *Bacillus subtilis* as well as the fungi *Saccharomyces cerevisiae* were not affected by nostocyclamide, when exposed at concentrations of up to 10 µM (Todorova & Jüttner,

1996). However, nostocyclamide was highly active against other cyanobacteria and green algae, with very low minimal inhibition concentrations (0.1 to 1.0 µM) (Todorova & Jüttner, 1996). This might provide a first hint about the ecological role of cyclamides in general: rotifers and crustaceans are important grazers of cyanobacteria and, as such, suitable target organisms. On the other hand, other phototrophic organisms are natural competitors for limited resources (Todorova & Jüttner, 1996). However, despite these first efforts on the investigation of their biological function, the mode of action of cyclamides, as well as their occurence in different cyanobacteria is still not elucidated. In our studies, aerucyclamides A-D were tested for grazer toxicity against *T. platyurus* **(Portmann*, et al.*, 2008a, Portmann*, et al.*, 2008b)** and parasitic protists (e.g. *Plasmodium falciparum* K1, *Trypanosoma brucei rhodesiense*) **(Portmann*, et al.*, 2008b)**.

2.1 Structural characterisation of microcystins

Isolation and structure elucidation of [D-Asp^3,(E)-Dhb^7]MC-RR from Planktothrix rubescens

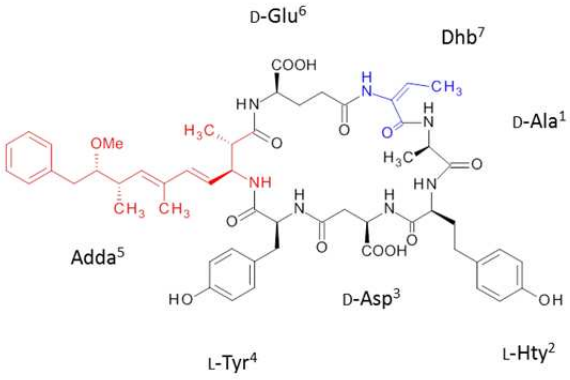
Planktothrix rubescens is a filamentous cyanobacterium that is frequently found in natural pre-alpine lakes. Many of these lakes are extensively used for recreation and drinking water supply, e.g. Lake Zürich in Switzerland. During the second half of the summer period this cyanobacterium yearly accumulates to very high densities in the deeper water layers (Micheletti*, et al.*, 1998). *P. rubescens* strain A7 (originally strain BC 8307, Bristol Collection) was isolated from Lake Zürich in 1993 by A.E. Walsby and was made axenic by extensive washings with sterile 50% diluted cyanobacterial medium **(Blom*, et al.*, 2001)**. Extracts of this particular strain were separated by high pressure-liquid chromatography (HPLC) into 14 fractions and tested for grazer toxicity in the *T. platyurus* 24-acute grazer toxicity assay. This crustacean serves as a benchmark organism for the evaluation of the ecotoxicological potential of natural products (Kurmayer & Jüttner, 1999). Only one of the toxic fractions showed the typical absorption spectrum for microcystins. Afterwards, preparative HPLC was applied to obtain these unknown microcystins in high amount. The toxin was identified as [D-Asp^3,(*E*)-Dhb^7]MC-RR **(Blom*, et al.*, 2001)** on the basis of several analytical characteristics: first, the purified microcystin had an absorption maximum at 239 nm in methanol and exhibited a quasi-molecular ion obtained by electrospray mass spectrometry at *m/z* 1024.6 $[M+H]^+$. The amino acid composition was determined using gas chromatography-mass spectrometry (GC-MS) after hydrolysis of the microcystin and subsequent trifluoroacetylation. Arg was determined after conversion of the guanidine group into the dimethylpyrimidyl derivative by acetylacetone (Mori*, et al.*, 1978). The stereochemistry of the amino acids of the hydrolysate was determined using Marfey's method for the separation of the enantiomers (Harada*, et al.*, 1995). Under the conditions applied, D-Asp, D-Ala, L-Arg and D-Glu were found. Adda decayed upon hydrolysis, and Dhb

was converted to lactate. NMR spectroscopy could confirm each amino acid residue in the corresponding spectra as well as the configuration of Dhb (determined to be *E*). The quasi-molecular ion obtained by electrospray mass spectrometry at *m/z* 1024.6 $[M+H]^+$ was consistent with three previously isolated microcystins: [D-Asp^3]MC-RR (Meriluoto*, et al.*, 1989, Sivonen*, et al.*, 1992, Luukkainen*, et al.*, 1993), [Dha^7]MC-RR (Sivonen*, et al.*, 1992, Luukkainen*, et al.*, 1993), and [D-Asp^3,(*E*)-Dhb^7]MC-RR (Sano & Kaya, 1995). Indeed, analyses of 1D and 2D ^{1}H NMR spectra provided evidence that the molecule in *P. rubescens* strain A7 is identical to that described by Sano and Kaya (Sano & Kaya, 1998). To determine the specific toxicity of a compound, the molar absorption coefficient is an essential parameter **(Blom*, et al.*, 2001)**. Usually, the value of ε = 39'800 l mol^{-1}cm^{-1}, originally determined for MC-LR (Harada*, et al.*, 1990) is used to determine the concentrations of microcystins. However, the molar absorption coefficient for [D-Asp^3,(*E*)-Dhb^7]MC-RR **(2)** was determined to be ε = 50'400 l mol^{-1}cm^{-1} in methanolic solution for the maximum at 239 nm **(Blom*, et al.*, 2001)** after hydrolysis of the microcystin and quantitatively measurement of the amino acids either with Marfey's method (and L-Ala as internal standard) or with GC-MS analysis (and methyldeuterated L-Ala as internal standard). Thus, the value determined for [D-Asp^3,(*E*)-Dhb^7]MC-RR was larger than reported for MC-LR, and it is assumed that the Dhb moiety contributes to this increase in the absorption at 239 nm **(Blom*, et al.*, 2001)**. Moreover, the observed difference of the molar absorption coefficients has a major influence on the assessment of microcystin-caused toxicity, which is usually done with the value of ε = 39'800 l mol^{-1}cm^{-1}. However, this latter value might underestimate the concentration of [D-Asp^3,(*E*)-Dhb^7]MC-RR in the water and illustrates the necessity to know the individual structures of microcystins.

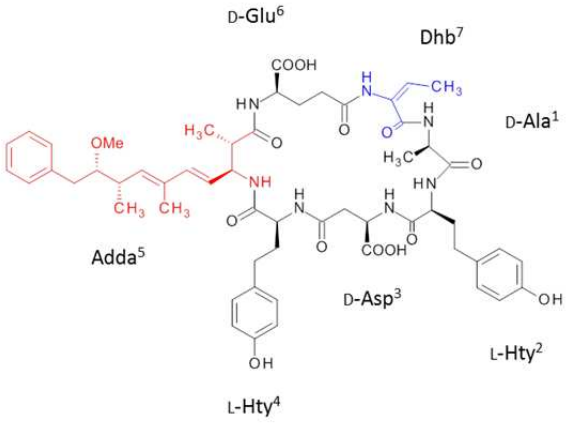
Isolation and structure elucidation of [D-Asp^3,(E)-Dhb^7]MC-HtyY and [D-Asp^3,(E)-Dhb^7]MC-HtyHty of Planktothrix rubescens

Recently, two new microcystin variants were isolated from *P. rubescens* strain No80 obtained from a specific lake (Schwarzensee) in the Austrian Alps (Upper Austria) **(Christiansen*, et al.*, 2008)**. Aqueous methanolic extraction followed by preparative HPLC yielded the two microcystins in milligram amounts for further analyses. Mass spectrometric analyses gave a quasi-molecular ion for the first compound of *m/z* 1074.4815 $[M+Na]^+$ suggesting a molecular formular of $C_{55}H_{69}N_7O_{14}Na$ (calculated 1074.4800, Δ -1.5 mmu). To determine the enantiomers of the amino acids, the highly purified microcystins were first esterified with 3 M HCL gas in MeOH and subsequently trifluoroacetylated. The analysis yielded D-Asp, D-Ala, D-Glu, L-Tyr, and L-Hty. The Adda unit could be readily assembled straight forward on the basis of NMR data; additionally, NMR data demonstrated the presence of Dhb in *E* configuration instead of Dha or Mdha commonly found in other microcystins. ROESY data helped to evaluate the final sequence of the first microcystin to be *cyclo*-(Ala^1-Hty^2-Asp^3-Tyr^4-Adda^5-Glu^6-Dhb^7). The second microcystin gave a quasi-molecular ion of *m/z* 1088.4960 $[M+Na]^+$ suggesting a molecular formula of $C_{56}H_{71}N_7O_{14}Na$ (calculated

1074.4800, Δ -0.3 mmu). Instead of Tyr and Hty the amino acid analysis revealed two Hty moieties in the molecule. In conclusion, extensive analytical studies revealed in this particular *P. rubescens* strain No80 two microcystin variants with rather unusual Hty at position 4 instead of Arg. The structures turned out to be [D-Asp^3,(*E*)-Dhb^7]MC-HtyY that contributed 76 ± 5% (SE) and [D-Asp^3,(*E*)-Dhb^7]MC-HtyHty, which made 7 ± 4% of the total microcystin content **(Christiansen, *et al.*, 2008)**.



[D-Asp^3,(*E*)-Dhb^7]MC-HtyY **(6)**



[D-Asp^3,(*E*)-Dhb^7]MC-HtyHty **(7)**

2.2 Structural characterisation of cyanopeptolins

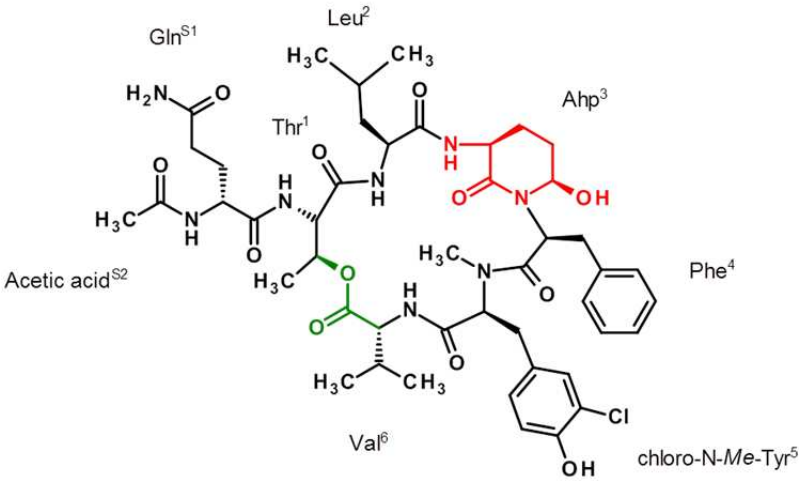
Isolation and structure elucidation of oscillapeptin J of Planktothrix rubescens

When isolating [D-Asp^3,(*E*)-Dhb^7]MC-RR from *P. rubescens* strain A7, a second but more hydrophilic compound was found that additionally exhibited strong acute toxicity in the bioassay with *T. platyurus*, comparable to the one of microcystins **(Blom, *et al.*, 2003)**. However, this fraction did not show features of a microcystin such as the typical spectrophotometric characteristics and source fragmentation patterns. The frozen *P. rubescens* biomass was treated with 60% aqueous methanol to extract the majority of the toxin. The unknown compound showed a strong absorption ad 200 nm (maximum), 220 nm (shoulder), and 278 nm (local maximum). With electrospray mass spectrometry, the compound exhibited a quasi-molecular ion a *m/z* 1093.2 $[M+H]^+$. The toxin was collected and purified in larger amount for subsequent assays and analytical analyses. High-resolution electrospray Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) of the sodium adduct gave a signal at 1115.43205 amu, corresponding to $[C_{47}H_{68}N_{10}O_{18}SNa]^+$ with a relative mass error of 0.5 ppm. Therefore, the uncharged compound had the formula $C_{47}H_{68}N_{10}O_{18}S$. In-source fragmentation of the electrospray ionisation (ESI) led to loss of 18

amu indicating hydroxyl amino acids, and 80 amu, indicating the presence of sulphate. Acid hydrolysis of the toxin and derivatization with *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) showed the presence of sulphate, *allo*-Ile, glycerate, Thr, *N*-methyltyrosine (MeTyr), and Tyr, the stereochemistry was determined via chiral GC-MS. Additionally, quantitative analysis showed the presence of two Thr subunits. Oxidation of the toxin with CrO_3 (Itou*, et al.*, 1999) followed by acid-catalysed hydrolysis led to the formation of L-Glu as the major degradation product of Ahp (Fujii*, et al.*, 2000). The peptide sequence of the unknown compound and absolute stereochemistry of Ahp as well as the lactone ring closure was deduced from NMR spectroscopy. Its molecular structure was determined to be a new oscillapeptin, which was named oscillapeptin J **(3) (Blom*, et al.*, 2003)**.

Isolation and structure elucidation of cyanopeptolin 954 from Microcystis NIVA Cya 43

When examine the extract of *Microcystis* NIVA Cya 43 with bioassay-guided fractionation two compounds with inhibitory activity against chymotrypsin were found **(von Elert*, et al.*, 2005)**. One compound was shown to be identical to nostopeptin BN920, previously isolated from the cyanobacterium *Nostoc*. However, the molecular formula of the new compound, named cyanopeptolins 954 **(8)** according to its molecular mass of 954 amu, was found to be $C_{46}H_{68}N_8O_{12}Cl$; the isotopic distribution of the ions indicated the presence of chlorine. Acid amino analysis and derivatization with MTBSTFA of the peptide revealed the following amino acids: glutamine (Gln), Leu, Val, *O*-acylated Thr, *N*-alkylated Phe, and *N*-methylated 3'-chloro-Tyr. Ahp was found after acid-catalysed hydrolysis as described above. To determine the configuration of the amino acids, the hydrolysate was acylated with trifluoroacetic acid anyhydride. The structure of *N*-methylated 3'-chloro-Tyr was deduced from the 2D NMR spectrum **(von Elert*, et al.*, 2005)** and confirmed by comparison of the NMR data with those published from scyptolins A and B (Matern*, et al.*, 2001) and micropeptins 478-A and –B (Ishida*, et al.*, 1997).



Cyanopeptolin 954 **(8)**

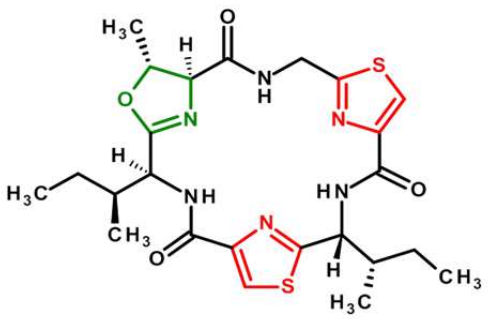
Isolation and structure elucidation of cyanopeptolin 1020 from Microcystis aeruginosa UV-006

When analysing the peptide pattern *of Microcystis aeruginosa* UV-006, originally isolated from the Hartebeerspoort Dam near Pretoria, South Africa **(Gademann*, et al.*, 2010)**, altogether nine compounds were found, which exhibited acute toxicity against *T. platyurus*. Among these compounds eight could be assigned to be known or new microcystins, however, one showed typical absorption spectrum of a cyanopeptolin. In order to elucidate the structure of this toxin, this fraction was purified, analysed by NMR spectroscopy and chemical degradation (such as amino acid analysis). The molecular formula was determined to be $C_{50}H_{72}N_{10}O_{13}$. The different (amino) acids were determined to be Glu, Thr, Arg, Ahp, Phe, N-MeTyr, Val, and hexanoic acid. According to its mass this compound was named cyanopeptolin 1020 **(4) (Gademann*, et al.*, 2010)**.

2.3 Structural characterisation of aerucyclamides

Isolation and structure elucidation of aerucyclamides A - D

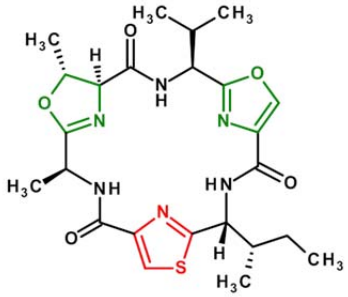
Aqueous methanolic extracts of *M. aeruginosa* PCC 7806 were investigated for the presence of bioactive compounds **(Portmann*, et al.*, 2008a, Portmann*, et al.*, 2008b)**. First, two lipophilic compounds were further investigated. The major peak, named aerucyclamide A **(5)**, displayed an exact mass of *m/z* 557.1982, which supported the molecular formula $C_{24}H_{34}N_6O_4S_2Na$ for the sodium adduct. This elemental composition was additionally corroborated by labelling experiments with ^{15}N- and ^{34}S, revealing the presence of six N atoms and two S atoms. NMR analyse showed typical peptide characteristics, and pointing to the presence of methyloxazoline (MeOzn), thiazoline (Tzn) and thiazole (Tzl), also on the basis of the presence of two sulphur atoms. The sequence of aerucyclamide A was unambiguously established by HMBC long-range correlations to be *cyclo*-(-glycin-Tzl-Ile-Tzn-Ile-MeOzn-)- **(Portmann*, et al.*, 2008a)**. The constitution of the second compound, aerucyclamide B **(9)**, revealed the difference of two H atoms when compared to aerucyclamide A **(5)**, suggesting that aerucyclamide B was and oxidative derivative of aerucyclamide A **(5)**. Indeed, MnO_2 in benzene could be used to convert aerucyclamide A **(5)** to aerucyclamide B **(9)** through oxidation (You & Kelly, 2004, Hughes & Moody, 2007), which was then shown to be identical to the natural sample by HPLC co-injection. HMBC spectroscopy allowed for



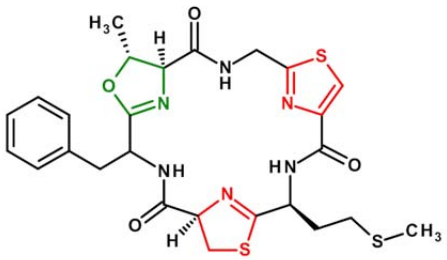
Aerucyclamide B **(9)**

the establishment of the sequence *cyclo*-(-glycin-Tzl-isoleucin-Tzl-isoleucin-MeOzn-)- **(Portmann*, et al.*, 2008a)**. Configuration of aerucyclamides A **(5)** and B **(9)** was assigned after chemical degradation. For aerucyclamide A **(5)** L-Thr and D-Cys were assigned, additionally, L-Ile and D-*allo*-Ile. The differentiation of L-isoleucine, L-*allo*-isoleucine, D-isoleucine, and D-*allo*-isoleucine was achieved by using gas-chromatography on chiral stationary phases. The exact position of L-Ile and D-*allo*-Ile in the molecule was determined as follows: amino acids adjacent to thiazole rings are not liberated under normal hydrolysis conditions; therefore, the L-Ile in aerucyclamide B **(9)** (found after acid hydrolysis) must be adjacent to the more hydrolytically labile oxazole ring system, and the D-*allo*-isoleucine between the two thiazole, respectively thiazole and thiazoline ring systems **(Portmann*, et al.*, 2008a)**.

In the second part of the study, aerucyclamides C **(10)** and D **(11)** were isolated and their structures established by extensive NMR-studies **(Portmann*, et al.*, 2008b)**. The molecular formula ($C_{24}H_{32}N_6O_5S$) of aerucyclamide C **(10)** was suggested by high resolution mass spectrometry, which was complemented by isotope labelling studies, establishing the presence of six N atoms and one S atom. Again, configuration of this compound was carried out after chemical degradation, derivatization of the amino acids, and analysis by



Aerucyclamide C **(10)**



Aerucyclamide D **(11)**

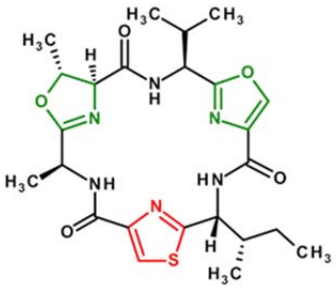
chromatographic methods. Beside D-*allo*-Ile, L-Ala, L-Val, and L-Thr were found, thus establishing the structure of aerucyclamide C **(10)**. Aerucyclamide D **(11)** displayed an exact mass of m/z 587.1569, thus suggesting the molecular formula $C_{26}H_{31}N_6O4_5S_3$. The sequence was unambiguously established by HMBC long-range correlations, hydrolysis and derivatization established the presence of L-Cys, L-Phe, L-Thr, and L-methionine (Met) **(Portmann*, et al.*, 2008b)**.

*Structure revision of microcyclamide 7806A **(12)***

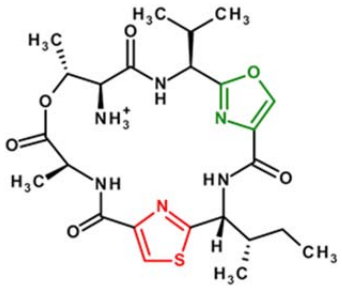
Recently, the biosynthetic origin of the cyclamides has been investigated by Dittmann and co-workers (Ziemert*, et al.*, 2008). These studies established ribosomal peptide synthesis

with hypervariable cassettes in a strongly conserved genetic background featuring tailoring enzymes. Based on the genetic data of *M. aeruginosa* PCC 7806, the same strain we used for our studies, four peptide sequences were found predicting the presence of four cyclamides with the amino acid sequences (following the one letter code): ATVSIC, FTGCMC, ITGCIC, and ITGCIC (Ziemert*, et al.*, 2008). Based on these molecular data two compounds, microcyclamide 7806A **(12)** and 7806B **(14)**, were isolated and characterized (Ziemert*, et al.*, 2008).

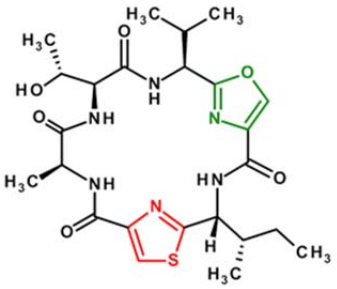
While the lipophilic aerucyclamides A-D eluted late on the analytical C18 column, the microcyclamides eluted earlier, and were thus more polar. In addition to this physical difference in polarity, NMR chemical shift data were reported that are unlikely to match the proposed structure (Ziemert*, et al.*, 2008). The structural identity of aerucyclamide C



Microcyclamide 7806A **(12)**
(original structure)
(identical to aerucyclamide C **(10)**)



Microcyclamide 7806A **(13)**
(revised structure)



Microcyclamide 7806B **(14)**

(10) and microcyclamide 7806A **(12)** on different spectroscopic data **(Portmann*, et al.*, 2008b)** led to a re-evaluation of the structural assignment of microcyclamide 7806A **(12).** Moreover, under laboratory conditions microcyclamide 7806A could be obtained by treating a sample of aerucyclamide C with 1% trifluoroacetic acid in H_2O for 12h. The subsequent NMR analyses for the synthetic sample of microcyclamide 7806A matched those reported before (Ziemert*, et al.*, 2008). Therefore, a new *revised* structure for microcyclamide 7806A **(13)** was proposed with an open oxazoline ring **(Portmann*, et al.*, 2008b)**. It is assumed that the isolation procedure applied by Dittmann and co-workers (employing trifluoroacetic acid) led to the formation of the two hydrolysis products microcyclamide 7806A **(13)** and 7806B **(14) (Portmann*, et al.*, 2008b)**. Interestingly, an inhibition in antiproliferative, antifungal, antibacterial, and cytotoxic assays for both microcyclamides were not observed, thus indicating that the ring-opened hydrolysis products likely lost their bioactivity potential. Finally, it is interesting to note that the four genetic sequences found (ATVSIC, FTGCMC, ITGCIC, and ITGCIC) encode for the four aerucyclamides **(Portmann*, et al.*, 2008b)**.

2.4 Toxicity of microcystins, and their inhibition of protein phosphatases

Toxicity of microcystin derivatives

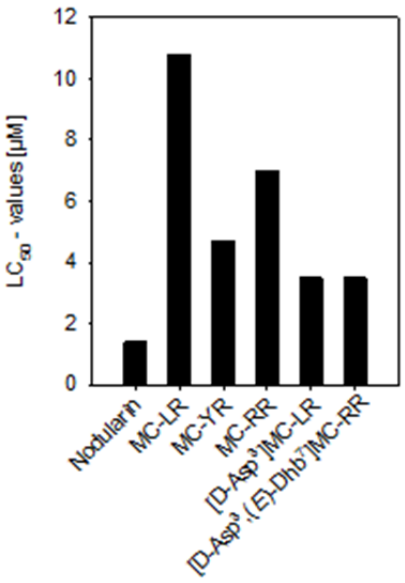


Fig. 1. LC_{50}-values of microcystin-derivatives and nodularin of their toxicity to *T. platyurus*. Taken from **Blom & Jüttner, 2005**, modified.

Purified [D-Asp^3,(*E*)-Dhb^7]microcystin-RR and [D-Asp^3]MC-LR, as well as commercial MC-RR, MC-YR, MC-LR, and nodularin were tested for grazer toxicity against *T. platyurus* in a 24-acute grazer toxicity assay **(Blom & Jüttner, 2005)**. Nodularin was the most toxic compound tested (LC_{50} = 1.4 µM), followed by [D-Asp^3,(*E*)-Dhb^7]MC-RR and [D-Asp^3]MC-LR (both LC_{50} = 3.5 µM), and MC-YR (LC_{50} = 4.7 µM) (Fig.1). MC-RR (LC_{50} = 7.0 µM) and MC-LR (IC_{50} = 10.8 µM) were less toxic in this assay compared to the other microcystins and nodularin. The higher toxicity of [D-Asp^3,(*E*)-Dhb^7]MC-RR as compared to MC-RR was also observed with the intraperitoneal (i.p). mouse bioassays: the LD_{50} of [D-Asp^3,(*E*)-Dhb^7]MC-RR was reported to be 250 µg/kg mouse (Sano & Kaya, 1998), while the LD_{50} of MC-RR exhibited much higher values of 500-800 µg/kg mouse (Painuly*, et al.*, 1988, Namikoshi*, et al.*, 1992).

Inhibition of protein phosphatases 1 and 2A by [D-Asp^3,(E)-Dhb^7]MC-RR

An explanation for the increased toxicity of [D-Asp^3,(*E*)-Dhb^7]MC-RR may be found in the replacement of the methylene group of the Mdha of the MC-RR by an ethylidene group of Dhb. The other changes of the molecular structure are not likely to contribute to its increased toxicity. It is believed that the Mdha-containing microcystins undergo a covalent binding with the protein phosphatases (Bagu*, et al.*, 1995). However, the removal of the double bond of methyldehydrobutyrine (Mdhb) in nodularin reduced the i.p. mice toxicity by more than a half (Namikoshi*, et al.*, 1993). Therefore, the inhibitory activity of nodularin (containing Mdhb) and five different microcystins (with Dhb or Mdha moieties) were tested and compared in a protein phosphatase assay using DiFMUP as substrate for PP1 and PP2A **(Blom & Jüttner, 2005)**. For this purpose, [D-Asp^3,(*E*)-Dhb^7]MC-RR and [D-Asp^3]MC-LR were isolated in high purity from *P. rubescens* and *M. aeruginosa*, respectively, and three other microcystins were purchased. After setting the optimal conditions of the assay such as enzyme and substrate concentration, all toxins were diluted in Tris-HCl (pH 7.0) to final concentrations in the range between 0.004-40 nM. Each dilution was prepared three times and each preparation was measured twice in three replicates (n=18 for each concentration;

n=9 for [D-Asp^3]MC-LR). The IC_{50} values differed essentially between the different microcystin congeners (Fig. 2). The data demonstrated that the Dhb–containing microcystin is a much weaker inhibitor of the protein phosphatases than compared to nodularin and Mdha-containing microcystins. The IC_{50} value when compared to the other microcystins was higher by a factor of 50 under the applied conditions. Generally, the inhibition of PP1 was weaker compared to PP2. However, [D-Asp^3,(*E*)-Dhb^7]MC-RR again exhibited the highest IC_{50}-value tested (Fig. 2). MC-LR, the most frequently observed microcystin in lakes (WHO, 1998) was the most active congener tested in the PP1 and PP2 assays. Apparently, the exchange from MeAsp by Asp in position 3 reduced the inhibitory activity only to a minor extent. However, the replacement of Dhb by Mdha in position 7 led to massive changes in the ability to inhibit PP2A **(Blom & Jüttner, 2005)**. This findings are supported by studies with motuporin, a nodularin related cyclic pentapepide showed that the replacement of Mdha by Mdhb no longer allowed the formation of a covalent bond between inhibitor and protein phosphatase (Bagu*, et al.*, 1997).

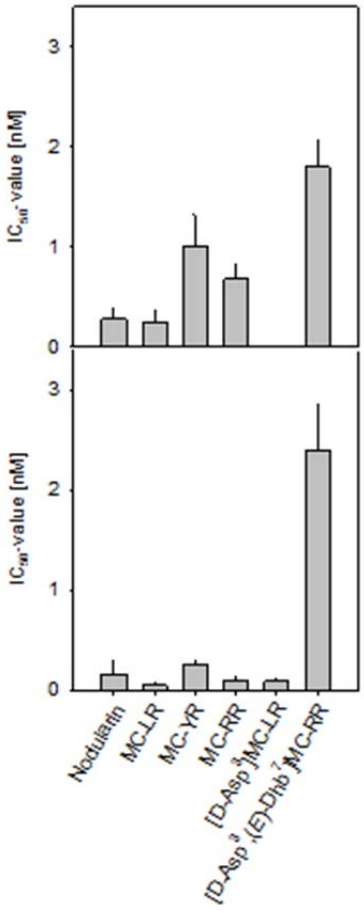


Fig. 2. IC_{50}-values of microcystin-derivatives and nodularin for their inhibition of PP1 and PP2A.Taken from **Blom & Jüttner, 2005**, modified.

In a consecutive study **(Hoeger*, et al.*, 2007)**, the inhibition potential of MC-LR, MC-RR, [Asp^3]MC-RR, nodularin and [D-Asp^3,(*E*)-Dhb^7]MC-RR was also compared by an anti-Adda-ELISA assay. The antibody used in the ELISA (enzyme-linked immunosorbent assay) recognizes the Adda-group of all compounds containing the Adda-side chain (microcystins and nodularin) (Fischer*, et al.*, 2001). All tested microcystin variants and nodularin were capable of inhibiting PP1 and PP2A. However, while MC-LR, MC-RR, [Asp^3]MC-RR and nodularin exhibited IC_{50} values in the very low nanomolar range, [D-Asp^3,(*E*)-Dhb^7]MC-RR inhibited PP1 10 to 20-fold less effectively. Moreover, MC-LR, MC-RR, and [Asp^3]MC-RR were among the most potent PP2A inhibitors tested so far with IC_{50} values in the upper picomolar range, whereas [D-Asp^3,(*E*)-Dhb^7]MC-RR with an IC_{50} value of 29.8 nM was distinctly less potent. These findings corroborated earlier results obtained with the fluorescent protein phosphatase assays using DiFMUP (Fig. 2) **(Blom & Jüttner, 2005)**.

In addition to the anti-Adda-ELISA assay, the covalent binding potential of the microcystins and nodularin was also analysed via in vitro incubation and Western blotting using antibodies against the catalytic subunit of PP1 and the Adda-group of microcystins and nodularin **(Hoeger*, et al.*, 2007)**. Covalent bindings could be identified with anti-PP1 or the anti-Adda antibody with MC-LR, MC-RR, and [Asp^3]MC-RR. In contrast,

no anti-Adda positive bands were detectable with [D-Asp^3,(*E*)-Dhb^7]MC-RR or nodularin and PP1, suggesting that none of these compounds bound covalently to PP1 **(Hoeger*, et al.*, 2007)**.

Our results were in congruence with earlier studies suggesting that Dhb-containing microcystins lacking the Mdha do not covalently bind to protein phosphatases, and likely pertains also microcystins with Ala or Ser at position 7 (Kaya*, et al.*, 2001, Hastie*, et al.*, 2005). However, as no weaker inhibition was reported for [D-Asp^3,(*E*)-Dhb^7]MC-HilR (Sano*, et al.*, 2004) and [D-Asp^3,(*E*)-Dhb^7]MC-HtyR (Hastie*, et al.*, 2005), it can be only speculated that the combination of the two arginines together with Dhb in [D-Asp^3,(*E*)-Dhb^7]MC-RR may be sterically hindering and thus restrict binding to the catalytic subunit of the protein phosphatases. However, while [D-Asp^3,(*E*)-Dhb^7]MC-RR was by far the weakest inhibitor of protein phosphatases, it was found to exhibit the highest toxicity in the *T. platyurus* bioassays (Fig. 1) **(Blom*, et al.*, 2001)**. This suggests, that the protein phosphate inhibiting capacity and the ability to form covalent binding does not consistently explain the observed toxicity. Moreover, the presence of Dhb may be additionally indicative for the tumor-promoting capacity of individual microcystins but not for its toxicity (Ichinose*, et al.*, 2002). Current risk assessment, which is entirely based on the toxicokinetic and toxicodynamic properties of MC-LR, seem to be not sufficient and should be reassessed (Dietrich & Hoeger, 2005).

2.5 Toxicity of cyanopeptolins, and their inhibition of serine proteases

Toxicity of cyanopeptolin derivatives

Up to seven variants of oscillapeptins have been isolated from different strains of *Oscillatoria agardhii* (Shin*, et al.*, 1995, Itou*, et al.*, 1999, Fujii*, et al.*, 2000), however, by that time oscillapeptin J was the first example of this type of peptides isolated from *Planktothrix* strains that exhibited acute grazer toxicity **(Blom*, et al.*, 2001)**. Oscillapeptin J showed toxicity against the freshwater crustacean *T. platyurus* (IC_{50} 15.6 µM), and, although slightly less toxic than [D-Asp^3,(*E*)-Dhb^7]microcystin-RR, the major toxin of *P. rubescens* strain A7 (see section 2.4), the acute toxicity was strong enough to contribute significantly to the total grazer toxicity of *P. rubescens* A7 **(Blom*, et al.*, 2003)**. In a second study, oscillapeptin J was tested against several aquatic organisms, however, toxicity was only found for crustaceans (*Eudiaptomus* sp. LC_{50} = 58 µM, *Daphnia* sp. LC_{50} = 203 µM). In addition to aquatic grazers, oscillapeptin J was tested on mice as model organism for vertebrates, however, without showing any effect up to a dose of 1000 µg/kg body weight. This clearly demonstrates that oscillapeptin J (in ecologically important concentrations of up to 200 µM) was only toxic for crustaceans, the most "dangerous" group of grazers of planktonic cyanobacteria **(Blom*, et***

***al.*, 2006)**. Likewise, cyanopeptolin 1020 was purified in high amount and tested against the freshwater crustacean *T. platyurus* **(Gademann*, et al.*, 2010)**. The LC_{50} value of 8.8 µM in the acute toxicity assay was comparable with the one of microcystins (Fig. 1) **(Blom*, et al.*, 2001, Blom & Jüttner, 2005)**. Thus, both cyanopeptolins, oscillapeptin J as well as cyanopeptolin 1020, showed grazer toxicity in the low micromolar range **(Blom*, et al.*, 2003, Gademann*, et al.*, 2010)**. The cyanopeptolins are known for the inhibition of digestive enzymes. However, this inhibition would cause starvation, likely slow death, but surely cannot explain the acute toxicity within 24 h **(Blom*, et al.*, 2006)**; thus, the acute toxicity of these congeners is still not understood **(Gademann*, et al.*, 2010)**.

Inhibition of serine proteases

Cyanopeptolins are known to inhibit digestive enzymes such as trypsin and chymotrypsin (**von Elert*, et al.*, 2005**, Yamaki*, et al.*, 2005). Both enzymes are typical digestive serine proteases, and high activities of trypsin- and chymotrypsin-like enzymes have been found in the gut system of crustacean grazers (von Elert*, et al.*, 2004). However, it seems that the inhibition of these proteases is mutually exclusive and solely dependent on the very specific amino acid at position 2 of the cyanopeptolins **(von Elert*, et al.*, 2005)**: hydrophobic amino acids in position 2, such as tyrosine (Tyr), phenylalanine (Phe), Leu, Hty or homoserine (Hse), are strong inhibitors of chymotrypsin. By contrast, basic amino acids at position 2 such as Arg or lysine (Lys) inhibit trypsin **(von Elert*, et al.*, 2005**, Yamaki*, et al.*, 2005). First, oscillapeptin J was tested against these proteases, and very high inhibitory activities were found for trypsin (IC_{50} = 20 nM), but also for chymotrypsin (IC_{50} = 2.8 µM), although by a two orders of magnitude lower extent **(Blom*, et al.*, 2006)**. Likewise, cyanopeptolins 954, as well as nostopeptin BN920 have been isolated und tested **(von Elert*, et al.*, 2005)**. Both derivatives inhibited chymotrypsin with 44.5 nM and 31.2 nM, respectively, which group these compounds into the strongest Ahp-containing chymotrypsin inhibitors tested so far. Additionally, cyanopeptolin 1020 was assayed against digestive enzymes, and exhibited a remarkable low IC_{50} value of 670 pM against trypsin **(Gademann*, et al.*, 2010)**. Moreover, cyanopeptolin 1020 was furthermore tested against several serine-proteases and low nanomolar values against human kallikrein (4.5 nM) and the blood coagulation factor XIa (3.9 nM) were determined **(Gademann*, et al.*, 2010)**. It can be speculated that the toxic effects described before might be generally the result of the inhibition of serine proteases others than the digestive enzymes, in particular as the large family of trypsin-like proteases are involved in may key processes related to the viability of organisms (Perona & Craik, 1997, Yousef*, et al.*, 2004). However, more detailed toxicological studies are needed to understand the toxic effects of cyanopeptolins **(Gademann*, et al.*, 2010)**.

In further experiments **(Blom*, et al.*, 2006)**, the adaptation of crustaceans to oscillapeptin J was investigated. If oscillapeptin J is produced as a grazer defence then it is conceivable that there may be adaptation of crustacean population that coexists with *P. rubescens* in its natural habitat. Both, *Eudiaptomus* sp. and *Daphnia* sp., were collected from Lake Walensee, an oligotrophic pre-alpine lake with no obvious occurrence of *P. rubescens*, and from Lake Hallwilersee, an eutrophic lake with annual mass developments of *P. rubescens* and exposed to different concentrations of the cyanopeptolin (Fig. 3). Applying statistical analyses it could be demonstrated that *Daphnia* sp. from Lake Walensee was significantly more affected than *Daphnia* sp. from Lake Hallwilersee. However, no significant differences were found for *Eudiaptomus* sp. **(Blom*, et al.*, 2006)**. The adaptation process that appears to have occurred in the *Daphnia* sp. population can be explained by the intense contact with this type of molecule as a result of filter-feeding that would favour adaptive response at a pheno- or genotypic level. Even more, 80% of the *Daphnia* population in Lake Hallwilersee were hybrids of *D. galeata* x *hyalina* (P. Spaak, personal communication). However, Lake Walensee was primarily dominated by *D. hyalina* (O. Köster, personal

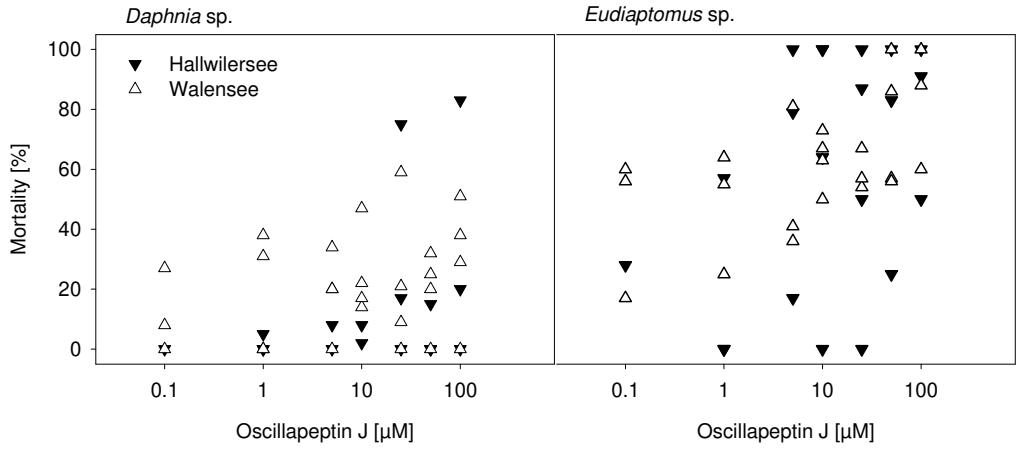


Fig. 3. Effect of different concentrations of oscillapeptin J on the mortality of Daphnia sp. (left panel) and Eudiaptomus sp. (right panel) from Lake Walensee (white triangles) and Lake Hallwilersee (black triangles).Taken from **Blom et al., 2006**.

communication), which might be itself already the result of an adaptation process. Previously, increased tolerance to microcystin-containing cyanobacteria by *D. pulicaria* has been reported (Sarnelle & Wilson, 2005). Adaptations to toxic compounds might be the result of structural changes of the target enzymes, the uptake and export by specific transporters, as well as detoxification reactions **(Blom*, et al.*, 2006)**. *Eudiaptomus*, however, is a selective feeder and is able to avoid ingestion of *P. rubescens* after recognition (Kurmayer & Jüttner, 1999). As oscillapeptin J is not released into the environment under normal conditions there is no need for *Eudiaptomus* sp. to develop an adaptive response **(Blom*, et al.*, 2006)**.

2.6 Toxicity of cyclamides

Biological evaluation of aerucyclamides A - D

Aerucyclamides A and B were tested for grazer toxicity against *T. platyurus* following published procedures **(Blom*, et al.*, 2001, Blom*, et al.*, 2003)**. Both compounds were found to be toxic to this sensitive crustacean with LC_{50} values of 30.5 and 33.8 µM, respectively **(Portmann*, et al.*, 2008a)**. Additionally, aerucyclamide C was tested against *T. playturus*, however, only low toxicity was found (LC_{50} value 70.5 µM) **(Portmann*, et al.*, 2008b)**. In the context of searching for lead structures against tropical infectious diseases from cyanobacteria, all four aerucyclamides were tested against the parasitic protists *Plasmodium falciparum* K1 and *Trypanosoma brucei rhodesiense* STIB 900, and against Rat Myoblast L6 Cells. The most active compound tested was aerucyclamide B, displaying a very low IC_{50} value against the chloroquine-resistant strain K1 of *P. falciparum* (IC_{50} = 0.7 µM). In addition, this compound displays a large selectivity for the parasite with respect to the L6 rat myoblast cell line, where an IC_{50} value of 120 µM was determined. Interestingly, the antiplasmodial activity is decreased by one order of magnitude by one structural modification from aerucyclamide B to aerucyclamide A, i.e., reduction of the Tzl to a Tzn residue. The most active compound against *T. brucei rhodesiense* was aerucyclamide C, albeit with moderate activity **(Portmann*, et al.*, 2008b)**. However, these first efforts on investigating the toxicity and growth inhibition of cyclamides have raised important questions regarding their mode of action on the cellular basis, but still lacking their possible ecological role in the natural environment.

2.7 *Outlook*: Metabolic characterisation of cryptic ecotypes in the toxic cyanobacterium *Planktothrix* spp.

Typically two types of *Planktothrix* strains are found in naturally occurring blooms, filaments with or without the ability to produce microcystins. A recent study on the ecological success of microcystin-negative mutants in bloom-forming *Planktothrix* populations from European lakes has shown that the abundances of inactive *mcy*-genotypes were linearly related to the total population density of *Planktothrix* (Ostermaier & Kurmayer, 2009). Microcystin-negative mutants were typically found in rather low proportions (mean, 6.5%), but the contribution of MC-negative mutants even exceeded 50% of the total population in one case. In view of the overall abundance and the omnipresence of the *mcy*-mutants in the studied lakes the authors concluded that the disadvantage of these MC-negative mutants due to the absence of microcystin should be considered of minor importance. In order to explain this counterintuitive finding it was hypothesized that a yet

undetected peptide (or class of peptides) might have functionally compensated the assumed disadvantage of the loss of microcystins. The search for hypothetically microcystins equivalents in *Planktothrix* species is depending on 1) the availability of a large number of strains and 2) and the knowledge about the genetic background of these strains (e.g. mutations in the *mcy* gene cluster). A metabolic characterisation of cryptic ecotypes in the toxic cyanobacterium *Planktothrix* spp. was carried out in close collaboration with Dr. R. Kurmayer (Austrian Academy of Sciences, Mondsee). Altogether 134 *Planktothrix* spp. strains collected from freshwater lakes from all over the world have been analysed with analytical methods (liquid-chromatography mass-spectrometry) for the presence of typical (e.g. microcystins and cyanopeptolins) and unknown cyanobacterial peptides. At the same time, all strains were phylogenetically analysed by molecular typing methods. To achieve high discrimination between the strains multilocus sequence typing was used. Thus, seven housekeeping genes and, additionally, the microcystin (*mcy*) gene were investigated. The combined data obtained from mass spectrometry and from the phylogenetic analyses revealed the presence of a unique peptide class with increasing percentage in microcystin-negative strains (Fig. 4). Currently, these data are summarized and prepared for publication.

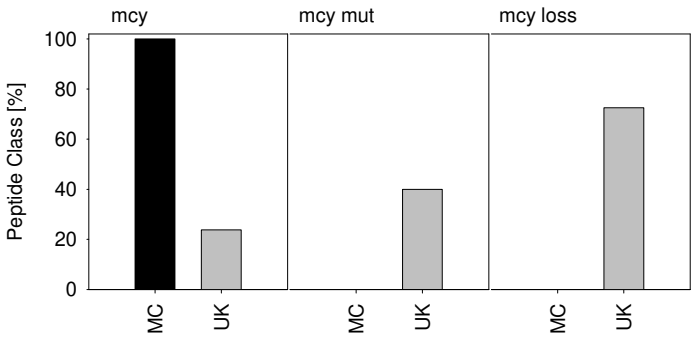


Fig. 4. Percentage of unknown peptide (UK) in *Planktothrix* strains that contain the *mcy*-cluster and producing microcystins (MC) (left panel), in strains that have a mutation in the *mcy*-cluster (middle panel), and in strains that lost the *mcy* cluster (third panel).

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3 Allelopathy - Growth Inhibitors/Promoters

3 Allelopathy - Growth Inhibitors / Promoters

Historically, allelopathy covers the interactions among different primary producers or between primary producers and microorganisms (Molisch, 1937). However, allelopathy can influence many ecological processes such as competition, community structure, or nutrient flow (Ervin & Wetzel, 2003), and it may additionally also modulate the interactions between prokaryotes, or between eukaryotes and bacteria **(Blom & Pernthaler, 2010)**. Allelopathic interactions of aquatic photoautotrophs consider compounds that are released into the environment to outcompete other species. For instance, studies carried out by Keating (Keating, 1977, Keating, 1978) have shown the dominance of pelagic cyanobacteria through the inhibition of diatom growth. However, allelopathy in pelagic environments depends strongly on sufficient production and excretion of the active compounds into the water (Gross, 2003); therefore, dilution might be the major limitation for in this type of chemical interaction (Lewis, 1986). On the other hand, aquatic ecosystems consist of littoral or benthic zones, where the competition for space is more severe and surface that can be colonized is rather limited (Gross, 2003). In these habitats one can assume high local concentrations of allelopathically active compounds produced by benthic photoautotrophs, which directly can act on targeted species (Gross, 2003).

Section 3.1 Potent algicides based on nostocarboline

Cyanobacteria are dominant in both pelagic and benthic freshwater habitats also due to their effective allelochemicals that inhibit the growth of competing cyanobacteria and algae (Gross, 2003). One of the first compounds isolated from the benthic cyanobacterium *Scytonema hofmannii* was named “cyanobacterin”, an effective allelochemical, inhibiting the growth of other cyanobacteria (Gleason & Paulson, 1984), eukaryotic algae (Gleason & Baxa, 1986), and higher plants (Gleason & Case, 1986) due to inhibition of the photosystem II. To inhibit the photosynthesis of competing organisms seem to be very effective, and this mode of action is widespread among cyanobacteria (Smith & Doan, 1999). We investigated the carbolinium alkaloid **nostocarboline**, isolated from the benthic cyanobacterium *Nostoc* 78-12A (Becher*, et al.*, 2005), and demonstrated its very high potential for the growth inhibition of competing phytoplankton organisms only (i.e., cyanobacteria and green algae **(Blom*, et al.*, 2006)**) and thus its high capability as antifouling agent **(Blom & Gademann, 2005)**. Studies on the mode of action of nostocarboline using chlorophyll-a fluorescence imaging demonstrated the light dependent inhibition of the photosynthetic electron transport, and thus mechanistically establishing the phytotoxic properties of this compound (Portmann*, et al.*, 2009).

Section 3.2 Allelopathic activity of the iron chelator anachelin

The reasons for the commonness of cyanobacterial dominance are in fact manifold (Dokulil & Teubner, 2000): cyanobacteria possess higher temperature optima compared to other algal groups (McQueen & Lean, 1987, Robarts & Zohary, 1987), superior uptake kinetics for inorganic carbon (low CO_2/high pH-hypothesis) (Shapiro, 1997), morphogenetic immunity to grazing by zooplankton (Porter, 1973, Burns, 1987, Lampert, 1987), and the capability to produce potent toxins affecting not only zooplankton but also other (Lindholm*, et al.*, 1989, Chorus & Bartram, 1999, **Blom*, et al.*, 2001**, **Blom*, et al.*, 2003**). Moreover, several hypotheses have been presented on how cyanobacteria suppress competing algae by secretion of allelopathic compounds (Murphy*, et al.*, 1976). The importance of phosphorous and nitrogen for algae and cyanobacteria has been well documented. Recently also the impact of iron on freshwater phytoplankton growth has found new interest (Behrenfeld*, et al.*, 1996, Butler, 1998, Hutchins*, et al.*, 1999). Although iron is one of the most abundant elements on earth, its bioavailability is very low due to the prevalence of insoluble iron oxide hydrate species (Neilands, 1981, Imai*, et al.*, 1999). Like other prokaryotes cyanobacteria have resolved this problem by producing small iron chelators called siderophores (Goldman*, et al.*, 1983, Wilhelm & Trick, 1994, Wilhelm*, et al.*, 1996).

Over the last 50 years, several hypotheses have been developed on how cyanobacteria dominate competing algae. First, Fogg and Westlake (Fogg & Westlake, 1955) suggested that the secretion of siderophores by cyanobacteria may have important effects on the growth of organisms in the water. Later on, this hypothesis was expanded by Tassigny and Lefèvre (Tassigny & Lefèvre, 1971), who speculated about an allelochemical activity of such secreted compounds. Later on it was shown that cocultivation of iron-limited cells of the pelagic cyanobacterium *Anabaena flos-aquae* and the chlorophyte *Chlamydomonas reinhardtii* resulted in the growth of the cyanobacterium only but not of the green algae. It was concluded that *Anabaena*-derived siderophores inhibited the growth of *Chlamydomonas* cells via uncharacterized toxicity (Matz*, et al.*, 2004). After extensive studies we provided evidence that the iron chelator **anachelin** (isolated from *Anabaena cylindrica*) operates likewise with a dual mode of action: this compound promoted the growth of *Microcystis aerugionosa* but, at the same time, reduced or even inhibited completely the growth of the green algae *Kirchneriella contorta*. Moreover, through the synthesis of derivatives, we were able to determine the structural elements required for this dual mode of action **(Blom*, et al.*, *Submitted*)**.

Section 3.3 Antibiotic effects of freshwater chrysophytes

Mixotrophic flagellates are common members of freshwater planktonic microbial assemblages (Bird & Kalff, 1986, Bennett*, et al.*, 1990) and their ecological role appears to be related to their dominant mode of nutrition (phagotrophy of phototrophy) (Rothhaupt,

1996, Rothhaupt, 1997). Thus, at the same time, they may be predators of bacteria, but also competitors for limiting nutrients (Thingstad*, et al.*, 1996). *Ochromonadaceae* have been long known for the production of allelopathic secondary metabolites. Allelochemical compounds have been extracted from the biomass of *Ochromonas* sp. already in the 70ies of the last century, which inhibited the growth of other microorganisms. However, the so far studied strains (e.g. pathogenic *Staphylococcus aureus*, *Bacillus megaterium*) are rather unlikely to coexist with the flagellates in their natural habitat. Therefore, **allelopathic effects** of various fractions of biomass extracts and culture supernatants of different ***Ochromonas/Poterioochromonas*** species were tested on freshwater bacterial isolates **(Blom & Pernthaler, 2010)**.

Section 3.4 Suboptimal light conditions negatively affect Planktothrix rubescens *but are beneficial for accompanying* Limnohabitans *spp.*

Allelopathic interactions are apparently enhanced under biotic or abiotic stress (Lovett*, et al.*, 1989, Tang*, et al.*, 1995, Reigosa*, et al.*, 1999). Complex field situations may have not only one but multiple stressors acting on the competing organisms, e.g. limited nutrients, ultraviolet radiation, or too high or too low light intensities. There is evidence that studies incorporating common stressors may resolve contradicting results on allelopathic activity (Gross, 2003). Moreover, it is very well known that cyanobacteria generally can affect the development of heterotrophic bacteria (Van Hannen*, et al.*, 1999, Van den Wyngaert*, et al.*, 2011). It was shown both that bacteria may benefit from cyanobacteria, e.g. by colonizing their mucilage and utilizing their extracellular compounds (Eiler*, et al.*, 2006), but also that others are negatively affected by cyanobacterial production of secondary metabolites with antibacterial properties (Østensvik*, et al.*, 1998). A *Microcystis* bloom might inhibit the growth of Betaproteobacteria related to *Limnohabitans* sp. (Horňák*, et al.*, 2008) but also stimulate the growth of members of *Alphaproteobacteria* (e.g. *Sphingomonas* sp.) (Park*, et al.*, 2001, Maruyama*, et al.*, 2003). We examined the effect of light on *P. rubescens* and on its accompanying bacteria, both *in situ* and in laboratory experiments **(Horňák*, et al.*, 2012)**. Our results suggest that *Limnohabitans* species in lakes might profit from the presence of physiologically stressed *P. rubescens*, likely by increase in nutrients through disintegrating filaments, whereas under optimal conditions cyanobacteria might inhibit their growth through production of allelopathic compounds.

Section 3.5 Production of the antifungal compound pyrrolnitrin in members of the Burkholderia cepacia complex

Although my research is generally focussed on the chemical interactions between aquatic microorganisms, I was very glad to collaborate with the group of Prof. L. Eberl (Department of Microbiology, Institute of Plant Biology, University of Zürich) to study the antifungal

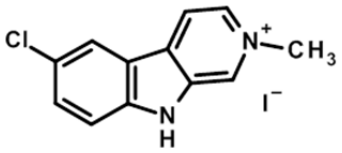
compound pyrrolnitrin in Burkholderia *species. These bacteria have been isolated from complete different habitats, e.g. soil, water, plants, insects, and even fungi. However, the species particularly protect important crops against fungal diseases belong to the so-called* Burkholderia cepacia complex *(BCC) and are from terrestrial origin (Vandamme, et al., 1997).*

During the last years it has become evident that bacteria do not only exist as individuals but often coordinate their activities in a process, which is known as "quorum sensing" (Fuqua*, et al.*, 1996, Whitehead*, et al.*, 2001). It was become evident that many gram-negative bacteria use *N*-acyl homoserine lactones (HSL's) to monitor their own population densities and to regulate a wide variety of functions, such as virulence, surface colonization, symbiosis and the production of antimicrobial compounds. Members of the genus *Burkholderia*, which are typically found in water and soil, are known for their ability to suppress fungal pathogens by the production of various antibiotic compounds. Compounds that have been shown to exhibit antifungal activity include lipopeptides (Kang*, et al.*, 1998), cepaciamides (Ying*, et al.*, 1996), cepacidines (Lee*, et al.*, 1994), siderophores (Thomas, 2007), altericidin (Kirinuki*, et al.*, 1984), pyrrolnitrin (Arima*, et al.*, 1964), glidobactins (Shoji*, et al.*, 1990), phenazines (Cartwright*, et al.*, 1995), and 2-hydroxymethyl-chroman-4-one (Kang*, et al.*, 2004). We investigated the role of quorum sensing in the regulation of the antifungal agent pyrrolnitrin in members of the genus *Burkholderia* **(Schmidt*, et al.*, 2009)**.

3.1 Potent Algicides Based On Nostocarboline

Biological evaluation of nostocarboline

Many photoautotrophic organisms excrete compounds into the environment that are able to effectively inhibit the growth of competing cyanobacteria and algae (Gross, 2003). Recently, the isolation, structure elucidation, and synthesis of a new quaternary carbolinium compound of *Nostoc* 78-12A was published, which was named nostocarboline **(15)** (Becher*, et al.*, 2005). Surprisingly, there are only few reports on carboline derivatives isolated from cyanobacteria, although alkaloids containing the carboline skeleton are often found in higher plants, animals, and bacteria (Bhat*, et al.*, 2005). Nostocarboline and seven derivatives thereof were prepared by chemical synthesis following modified protocols from the literature (Nakano*, et al.*, 2000, Ponce*, et al.*, 2003). All compounds were tested for growth inhibition of two different cyanobacteria, the toxic *M. aeruginosa* PCC 7806 and the nontoxic *Synechococcus* PCC 6911, and against the eukaryotic chlorophyte *K. contorta* SAG 11.81.



Nostocarboline **(15)**

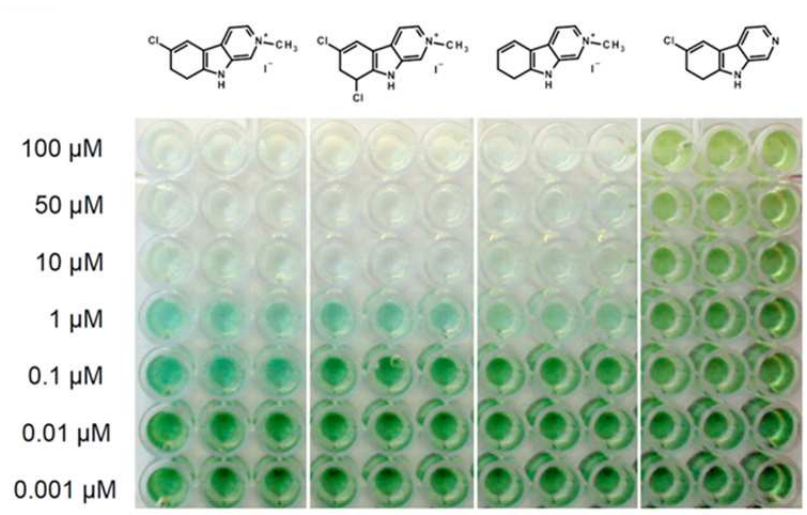


Fig. 5. Example of the experimental setup in a 96 well plate. Four compounds are shown, tested in triplicate in different concentrations (nostocarboline, 8-chloro-nostocarboline, deschloronostocarboline, and 6-chloronorharman (from left to the right).

Growth curves were measured over a period of 280 h. Two values were determined at 170 h after addition of different bioactive compounds: (I) the minimal inhibitory concentration (MIC), at which reduction of growth compared to the control was observed, and (II) the minimal phytotoxic concentration (MPC) at which reduction of growth compared to the time of addition of toxic compounds was observed. Nostocarboline was shown to be a potent inhibitor of cyanobacterial and algal growth (MIC = 1 µM against three photoautotrophic organisms tested) and induced rapidly growth inhibition of cyanobacteria at 10 µM within 24 h. A change in the phenotype of *Synechococcus* (bleaching of the cells, Fig. 5) could already be observed at low nanomolar concentrations of nostocarboline. IC_{50} values for the inhibition of the growth of the different phytoplankton species were determined to be IC_{50} = 2.1 µM (*M. aeruginosa*), IC_{50} = 5.8 µM (*Synechococcus*), and IC_{50} = 29.1 µM for (*K. contorta*). This clearly showed the high sensitivity of the toxic *M. aeruginosa* to nostocarboline. Structure-activity relationship (SAR) studies revealed that the quaternary group appears to be essential for biological activity against photoautotrophs, as the demethylated compounds (6-chloronorharmane and 6,8-dichloronorharmane) were found to be inactive (Table 1). The degree of chlorination had only minor impact on the biological activity of nostocarboline. However, the replacement of the substituent on the 2-N atom by either a benzyl- or an allyl-group led to an increase in activity compared to nostocarboline itself. It is interesting to note that all compounds induced rapid death of cells above MPC.

Moreover, nostocarboline was also tested against the growth of its producer, *Nostoc* 78-12A, and showed a strongly reduced activity (MIC value of 50 µM, MPC value of 100 µM compared to the other cyanobacteria). The large difference between the values for autoinhibition and for competing organisms likely results in sustained growth of the producing organisms with concomitant killing of competing autotrophs. Thus, the results point to the ecological role of nostocarboline by inducing competitive advantage via secretion of the bioactive compound. Additionally, growth experiments were performed in the dark. Virtually no inhibitory action was observed for concentrations up to 50 µM, indicating that nostocarboline activity is targeted to the photosynthetic activity of *M. aeruginosa*. Next, the nostocarboline-ciprofloxacin-hybrid was tested, as this quinolone

chimera could most likely possess a dual mode of action. It could be shown that this compound could retain the broad activity of nostocarboline against photoautotrophs, but additionally, gained activity against eukaryotic organisms, when compared to ciprofloxaxin itself. Moreover, this hybrid showed antibacterial activity against several bacterial strains in the lower micromolar range. Some of the reduced activity compared to ciprofloxacin might be explained by efflux phenomena, as the highest activity (0.7 µM) was found in an efflux deficient *E. coli* strain.

Table 1. Biological effect of nostocarboline, different nostocarboline derivatives, and ciprofloxacin against the growth of *M. aeruginosa* (M.a.), *Synechococcus* (Syn.), and *K. contorta* (K.c.).

	MIC [µM][1]			MPC [µM][2]		
Compound	M.a.	Syn.	K.c.	M.a.	Syn.	K.c.
Nostocarboline (11)	**1**	**1**	**1**	**10**	**10**	**>100**
6-chloronorharmane	>100	>100	nd[3]	>100	>100	nd[3]
6,8-dichloronorharmane	>100	>100	nd[3]	>100	>100	nd[3]
deschloronostocarboline	10	1	nd[3]	10	50	nd[3]
Allyl-derivative of (11)	10	1	nd[3]	10	10	nd[3]
Benzyl-derivative of (11)	10	0.1	nd[3]	10	10	nd[3]
8-chloronostocarboline	10	1	nd[3]	10	10	nd[3]
nostocarboline - ciprofloxaxin-Hybrid	1	1	10	1	50	10
ciprofloxacin	1	10	>100	>100	>100	>100

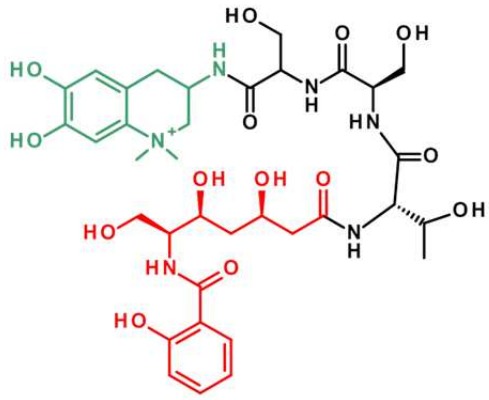
[1]MIC = minimal inhibitory concentration, at which reduction of growth compared to control could be observed; [2]MPC = minimal phytotoxic concentration, at which reduction of growth compared to the time of addition was observed; [3] nd= not determined.

The benefits of nostocarboline include (a) potent and fast reduction of photoautotrophs, (b) cheap and simple preparation, (c) the biogenic nature offering benefits in its potential registration ("natural algicide"), (d) selectivity to photosynthetic organisms, and (e) a structure which is amenable to easy modification resulting in more potent derivatives. For all these reasons, nostocarboline can thus be considered a promising lead structure for the development of algicides addressing the worldwide need for effective, biogenic, and simple antifouling agents. The results obtained by this study **(Blom*, et al.*, 2006)** were summarized in a patent **(Blom & Gademann, 2005)**.

3.2 Allelopathic activity of the iron chelator anachelin

Effet of the cyanobacterial siderophore anachelin on Microcystis aeruginosa

The cyanobacterial siderophore anachelin **(16)**, originally isolated from *A. cylindrica* (Beiderbeck*, et al.*, 2000) was synthetically produced in high amount (Gademann & Bethuel, 2004a, Gademann & Bethuel, 2004b, Gademann*, et al.*, 2007). It was shown to be free of side products, which might potentially contaminate natural products, e.g. cyanobacterial toxins. First, the allelopathic properties of anachelin were investigated on the toxic bloom-forming cyanobacterium *M. aeruginosa* PCC 7806 (**Blom*, et al.*, *Submitted***). Unequivocally, anachelin promoted the growth of the cyanobacterium in a dose-dependent manner. Already 800 nM were sufficient to yield a significant higher growth (measured as optical density at $OD_{675\ nm}$). This beneficial effect was independent of the two tested iron concentrations in the medium (0.1 and 10 µM), but the effect decreased with increasing iron concentration due to general higher growth of *M. aeruginosa* (Fig. 6; left panel). Presumably, the ability to bind iron by the siderophore resulted in higher



Anachelin **(16)**

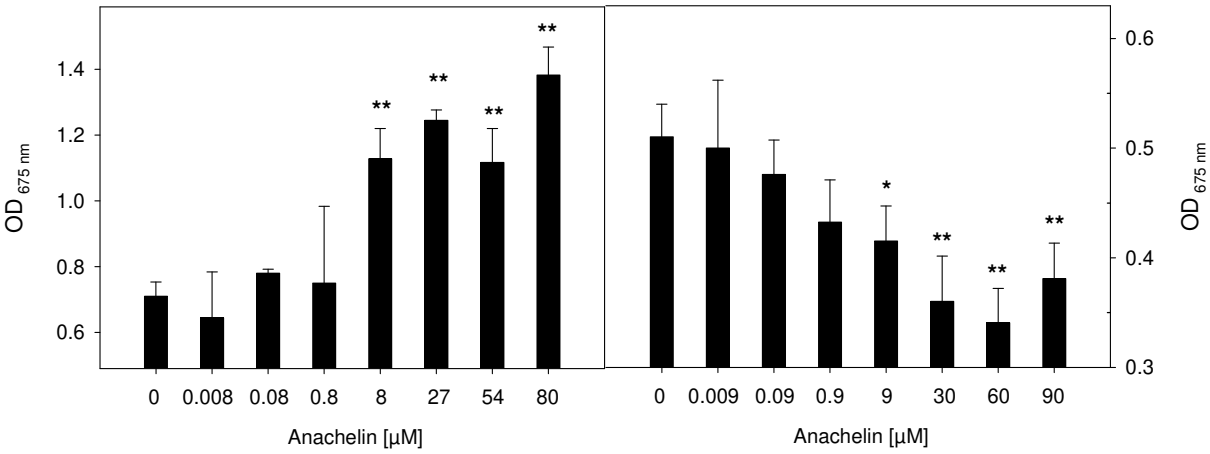


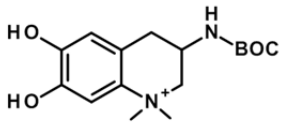
Fig. 6. Growth of *M. aeruginosa* (left panel) and *K. contorta* (right panel) after addition of seven different concentrations of anachelin, both grown in 10 µM FeCitrate. Error bars indicate the standard deviation of three replicates. Asterisks above bar indicate that the concentration of anachelin significantly changed the growth of the target organism compared to the control (* $p < 0.05$; ** $p < 0.01$; tested by ANOVA, followed by Dunnett's post hoc tests).

bioavailability and thus higher growth. *M. aeruginosa* itself is known to produce hydroxamate-type siderophores (Nagai*, et al.*, 2007). However, uncultured bacteria were shown to be able to grow by uptake of siderophores of distantly related bacteria (D'Onofrio*,*

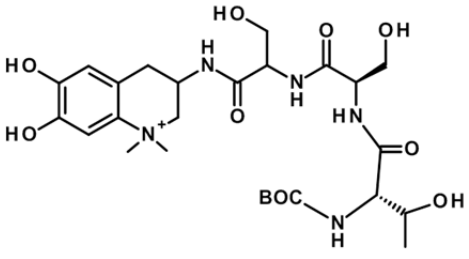
et al., 2010), a phenomenon which is known as 'siderophore piracy' (Schubert*, et al.*, 1999). Moreover, it seems to be quite common that the number of siderophore uptake genes in the genome of some bacteria is much higher than the number of synthesis genes (Moon*, et al.*, 2008). It is conceivable that such 'siderophore piracy' might also take place in cyanobacteria, although the exact mechanisms of iron delivery (either directly or through a specific transporter into the *Microcystis* cell) are still not known.

Effect of anachelin, its fragments and diastereoisomers on Kirchneriella contorta

We investigated the allelopathic activity of anachelin on a competing planktonic green alga *K. contorta*. In contrast to the effect on *Microcystis*, anachelin inhibited the growth of the green alga in a dose-dependent manner, again starting at nanomolar concentrations (Fig. 6, right panel). However, growth inhibition was observed at high surplus of iron in the medium (10 µM), indicating that the allelopathic effect was likely independent of iron availability, and thus questioning the 'iron monopoly' hypothesis (Murphy*, et al.*, 1976). Similarly, it was shown that siderophores from *Anabaena flos-aquae* inhibited the growth of *Chlamydomonas reinhardtii* via uncharacterized toxicity (Matz*, et al.*, 2004). In order to identify the structural requirements of this effect, three different fragment derivatives of anachelin were synthesized and tested for allelopathic activity. First we investigated the function of the quaternary ammonium cation (**(16)**; shown in green), which is known for many biological activities including antibacterial and antifungal properties (McDonnell & Russell, 1999, Vasilev*, et al.*, 2009). For this purpose, two fragments were synthesized containing the quaternary ammonium group such as **(17)** and **(18)**. In order to block the otherwise free terminal amino group, the chemically inert butyloxycarbonyl (Boc) group was introduced in both fragments. Fragment **(17)** was able to inhibit *K. contorta*, albeit to a very low extent. Growth could partially be repressed only at very high concentrations (60 µM). Virtually the same effect could be observed using anachelin already at two orders of magnitude lower concentrations. Moreover, fragment **(18)** did not have any negative impact on the growth of *K. contorta*, also at the highest tested concentrations (80 µM). Comparing the two fragments it is likely that the impact of the quaternary ammonium, when bound to the tripeptide as in fragment **(18)**



(17)



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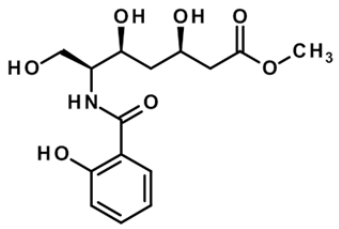


(19)

Anachelin fragments

is negligible for the overall growth inhibitory activity of anachelin. Similarly to quaternary ammonium ions polyketides (**(16)**; shown in red) are also known to possess broad biological activities (Jones*, et al.*, 2010). Thus, fragment **(19)** was prepared and tested against *K. contorta*. The allelopathic effect of anachelin could be totally reproduced by the polyketide fragment, which showed already significant impact at low micromolar concentrations. To investigate whether the allelopathic effect was unrelated to iron binding, two diastereoisomers were synthesized that are still able to bind iron but differ in the stereochemistry of the polyketide fragment. Interestingly, both 3,5,6-*epi*-anachelin and the 3,5-*epi*-anachelin were hardly affecting the growth of *K. contorta*. Thus clearly demonstrates the importance of the relative configuration for the allelopathic activity, and that the observed growth inhibitory activity is unrelated to iron binding and siderophore activity (Blom*, et al.*, *Submitted*).

The dual mode of action of anachelin

These results provide strong evidence on a molecular level that the siderophore anachelin possesses a dual mode of action, both promoting the growth of cyanobacteria and concomitantly inhibiting the growth of competing green alga. Anachelin can be considered a product of mixed polyketide synthase and non-ribosomal peptide synthetase pathways (Gademann, 2006), and it can be speculated that these fragments, and thus the corresponding activities, have biosynthetically merged over time to one single compound. These experimental findings for a cyanobacterium are complemented by dual mode of action hybrids (sideromycins) isolated from bacteria, where both iron chelating and antibiotic fragments are complementary present (Bickel*, et al.*, 1960, Knüsel & Nüesch, 1965, Braun & Braun, 2002). In an ecological context, it should be pointed out that the producer of anachelin, *A. cylindrica*, is a filamentous freshwater cyanobacterium that can form biofilms and can occur also as planktonic organism in ponds and small lakes (Becher*, et al.*, 2005). In such densely populated environments, where cooperative behaviour may be successful, secreted iron chelators are likely to be taken up by cells of same and other *A. cylindrica* filaments thus increasing the overall amount of bioavailable iron. At the same time, the anachelin that is lost by diffusion can be predicted to display a secondary benefit by inhibiting the growth of competing organisms such as chlorophytes and possibly other eukaryotic algae. By using one secondary metabolite, *A. cylindrica* can address two main challenges related to phytoplankton growth, *i.e.* nutrient limitation and inhibition of competitors **(Blom*, et al.*, *Submitted*)**.

3.3 Antibiotic effects of freshwater chrysophytes

Bioactivity of Ochromonadaceae

Mixotrophic *Ochromonadaceae* are known for the production of diverse bioactive compounds. Firstly, high mortality of different zooplankton species have been described after ingestion of different *Ochromonas* as well as *Poterioochromonas* species (Leeper & Porter, 1995, Boxhorn*, et al.*, 1998, Boenigk & Stadler, 2004). However, more detailed studies, as well as structure analyses of these toxins are still lacking. Additionally to toxins, which are stored inside the cells, bioactive compounds might be secreted into the environment that may affect aquatic organisms even when the flagellates themselves are not ingested (Boxhorn*, et al.*, 1998). Some of these effects have been studied with strains such as *Staphylococcus aureus*, *Bacillus megaterium*, which are likely not sharing the same natural habitat as *Ochromonas*. Considerably lesser attention has been paid to possibly differentially produced bioactive compounds in view of the pronounced physiological differences of flagellates during photo- and phagotrophic growth (Rothhaupt, 1997). Therefore, laboratory studies were conducted to give first insight into the antibiotic effect of three physiologically different ochromonads on freshwater bacterial isolates. Allelopathic effects of lipophilic and hydrophilic were examined in agar diffusion assays and in bacterial batch culture experiments. Experiments were conducted to test the influence of the nutritional mode (photo-or phagotrophic) on the production of bioactive compounds.

Antibiotic activity of biomass extracts from light-adapted chrysophytes

Freshwater bacteria belonging to different phylogenetic groups were isolated during summer/autumn months from the surface waters of Lake Zürich and Egelsee (canton Aargau, Switzerland), either after addition of nutrient rich medium, glucose or flagellate grazers. Partial 16S rRNA sequences of the isolated strains were obtained after DNA extraction and sequencing, following well established protocols as described before **(Blom & Pernthaler, 2010)**. Additionally, *Flectobacillus major* DSMZ 103 was used in some experiments (obtained from the German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). *Poterioochromonas* sp. strain DS (formerly known as *Ochromonas* sp. strain DS (Hahn & Höfle, 1998, Boenigk*, et al.*, 2006) were made axenic by adding an antibiotic mixture (chloramphenicol, streptomycin, gentamycin; ratio 1:1:05) according to protocols (Corno & Jürgens, 2006). Two more chrysophyte strains (*Ochromonas danica* SAG 933-7, and *Poterioochromonas malhamensis* SAG 933-1a) were used (purchased at the Culture Collection of Alga (SAG), Göttingen, Germany). All flagellate cultures were kept either in the light (subsequently referred to as 'light-adapted cultures') without addition of food bacteria, or in the dark fed with heat-killed *F. major* DSMZ 103 ('dark adapted culture')

in Jüttner-Friz-medium (Jüttner & Friz, 1974). Aqueous and methanolic extracts of light adapted flagellate biomass were produced by suspending freeze-dried biomass first in distilled water, and, after a centrifugation step, in methanol. Equivalent of 2 mg of freeze dried biomass were added to sterile filter discs, placed on agar plates, and covered with thin layer of bacterial culture. Areas of the inhibitions zones were calculated from the measured distance between the edge of the filter discs and the margin of visible bacterial growth. No visible inhibition zones were observed in any control treatments (distilled water or methanol) **(Blom & Pernthaler, 2010)**.

Generally strong differences were observed between the aqueous and methanolic extracts. The aqueous extracts of the biomasses of all three flagellates inhibited only the three bacterial strains related to *Flectobacillus* (strains Z013, Z021, and *F. major* DSMZ 103 (Fm103)), and Z021 was the most sensitive strain tested (Fig. 7). However, the inhibitory effect caused by the methanolic extracts was generally much stronger, but again the *Flectobacillus* strains showed highest growth inhibition (Fig. 7). *Sphingobium* sp. strain Z007, *Janthinobacterium* sp. strains E106 and 107, and *Azospirillum* sp. strain Z012 showed less distinct inhibition zones, whereas *Pseudomonas* sp strain E104 and *Acidovorax* sp. strain Z022 were hardy affected. Interestingly, extracts obtained by *Ochromonas danica* caused always the most pronounced effects (Fig. 7).

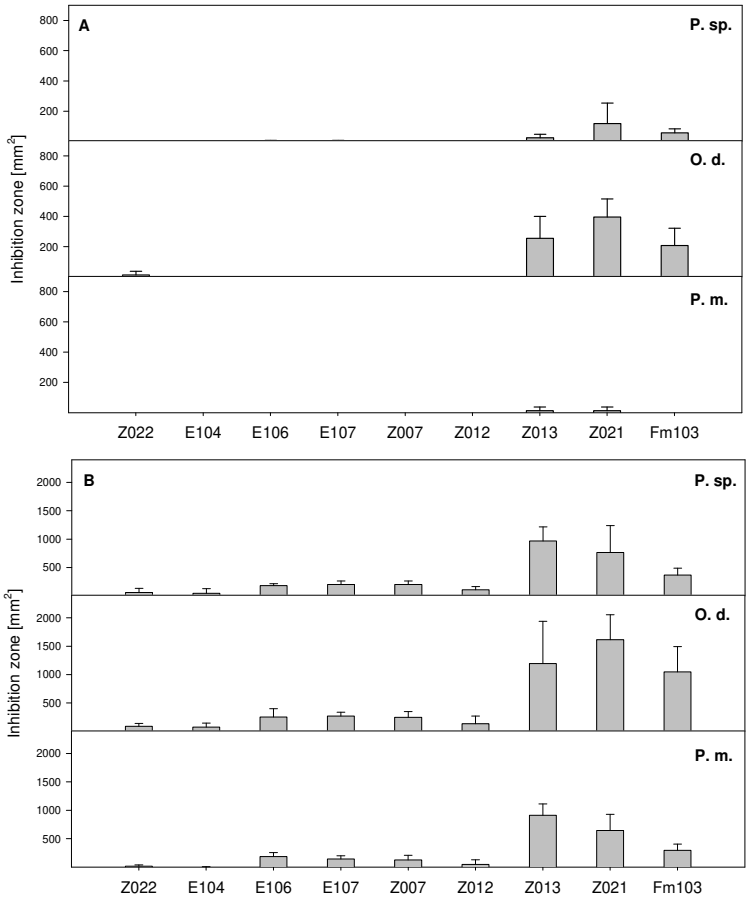


Fig. 7. Inhibition areas on agar plates [mm^2] induced by aqueous extracts (A) and methanolic extracts (B) of freeze dried cells of *Poterioochromonas* sp. (P.sp.), *O. danica* (O.d.), and *P. malhamensis* (P.m.) using different freshwater bacterial isolates. The error bars indicate the standard deviation of triplicate experiments. From: Blom & Pernthaler, 2010.

So far relatively few studies deal with the antibacterial effect of freshwater photo- or mixotrophs (Jones, 1988) on bacteria. The antibiotic effect of biomass implies storage of the bioactive compound in the flagellate cell, on first sight inaccessible for bacteria in the surrounding waters. However, numerous processes in the plankton might lead to the destruction of flagellate cells, e.g. viral lysis, bacterial attack or zooplankton grazing (Imai*, et al.*, 1993, Massana*, et al.*, 2007), resulting in the liberation of such compounds. Although

some flagellate cells might be lost, this could nevertheless be advantageous for the survival on the population level (Pohnert*, et al.*, 2007). Moreover, moderate lipophilic solvents such as methanol extract low-molecular weight compounds that can be transferred through intact cell membranes by direct cell-cell contact (Gross, 1999), and such compounds have been found before in marine algae (Steemann Nielsen, 1955, Sastry & Rao, 1994).

Inhibitory activity of photosynthetic pigments

It hast been suggested that chlorophyll derivatives such as chlorophyllides built the basis of the antibacterial substances and might play an important role in aquatic environments (Jørgensen, 1962). Therefore, methanolic extracts of light- as well as dark-adapted flagellate cultures were separated by liquid chromatography using a linear gradient with solvents composed of methanol:UV-treated distilled water (80:20) and methanol:acetone (80:20) **(Blom & Pernthaler, 2010)**. The pigment-containing fractions obtained by the extracts of the light-adapted flagellates were collected. Highest amount of chorophyll and chlorophyll derivatives were found in *O. danica*, followed by *Poterioochromonas* sp. strain DS, and last by *P. malhamensis*, indicating that *O. danica* might be regarded to gain more profit from autotrophy than the two *Poterioochromonas* strains **(Blom & Pernthaler, 2010)**. Likewise, fractions from the dark-adapted cultures (hardly containing any chlorophyll derivatives) were collected at the same retention time slots as for the light adapted cultures. Afterwards, all fractions were tested for inhibitory activity against *F. major* DSMZ 103. However, growth inhibition caused by HPLC fractions from the dark-adapted culture was at least as strong as the inhibition by the pigment-containing fractions, in most cases even stronger. Therefore, other compounds, which might be even more produced in higher concentrations in the dark and eluting at the same time as chlorophyll derivatives, were made responsible for the antibacterial effect against *F. major* DSMZ 103.

Antibiotic activity of supernatants or light- and dark-adapted chrysophytes

Additionally to extracts of biomasses, the supernatants of flagellate cultures were tested for the presence of antibacterial substances as well in agar diffusion assays using the strain *F. major* DSMZ 103. Moreover, the supernatants obtained by light-adapted cultures of *O. danica* showed the highest inhibitory activity as well, followed by *Poterioochromonas* sp. strain DS (Fig. 8). Hardly any effects were found for supernatants of *P. malhamensis*. Interestingly, the effect of 100 µl obtained either from light-adapted *O. danica* or dark-adapted *Poterioochromonas* sp. strain DS were comparable (Fig. 8).

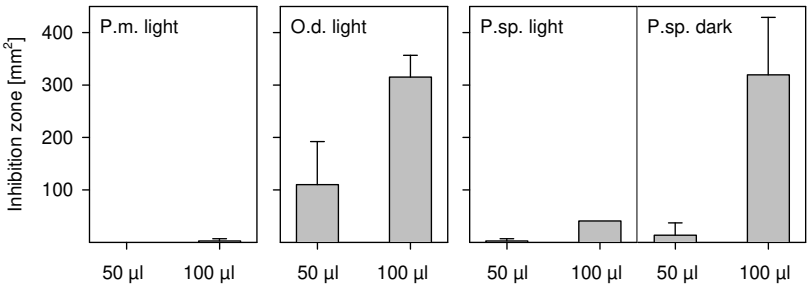


Fig. 8. Inhibition areas [mm^2] of *Flectobacillus major* DSMZ 103 induced by supernatants of different flagellate cultures: light adapted *P. malhamensis* (P.m.), *O. danica* (O.d.), *Poterioochromonas* sp. strain DS (P.sp. light), and dark adapted *Poterioochromonas* sp. strain DS (P.sp. dark). The error bars indicate the standard deviation of triplicate experiments. From: **Blom & Pernthaler, 2010**.

Additionally to the agar-diffusion assays growth inhibition of bacterial strains were tested in liquid culture, amended with either hydrophilic or lipophilic compounds obtained from supernatants of differently treated flagellate cultures. Supernatants from light- and dark-adapted cultures of *Poterioochromonas* sp. strain DS were applied to a C18 cartridge. The eluate was collected, evaporated, re-dissolved in medium and used for the subsequent bioassays as 'hydrophilic' fraction. Compounds still bound to the cartridge were washed out first with 10% aqueous (aq.) methanol, followed by 50%, and last by 100 % aq. methanol. The latter fraction was used after evaporation and suspending in medium again as 'lipophilic' fraction. Growth of five bacterial strains in these fractions was compared with the growth in unamended medium (control) using an absorption plate reader at 585 nm after subtracting the blank (medium filled cuvettes only). In addition, different aq. methanolic fractions of the cartridge itself were tested for growth inhibition, but without any observed effect. Growth rates were estimated from the slopes of the linear regression over those data points for which the cultures were in the exponential growth phase (log OD vs. time). Moreover, the lipophilic fraction of the dark-adapted flagellate culture was diluted with medium to various extents and tested on the sensitive strains *Flectobacillus* sp. strain Z013 and *F. major* DSMZ 103. Total biomass was calculated as the area underneath the growth curves generated over 48 h and compared to control treatment (growth of bacteria in unamended medium). Statistical analyses were used to test the differences between individual treatements for significance as described in detail elsewhere **(Blom & Pernthaler, 2010)**.

The hydrophilic compounds of the supernatants from both light- and dark-adapted flagellate cultures only affected the growth of *Sphingobium* sp. strain Z007 and *F. major* DSMZ 103. However, the growth of all bacteria tested was completely inhibited by the presence of lipophilic compounds from the supernatant of the dark-adapted culture of *Poterioochromonas* sp. strain DS, except *Pseudomonas* sp. strain E104, which showed only weak inhibition (Fig. 9). Lipophilic compounds from the light-adapted culture showed most distinct effect on *Sphingobium* sp. strain Z007 and *F. major* DSMZ 103, and less pronounced on *Janthinobacterium* sp. strain E106 and *Acidovorax* Z022. The dilution of the lipophilic fraction by 1:20 (or less) significantly reduced biomass of both *Flectobacillus* strains tested. However, at higher dilutions significantly decreased biomass was only observed in *Flectobacillus* strain Z013 (up to 1:10'000).

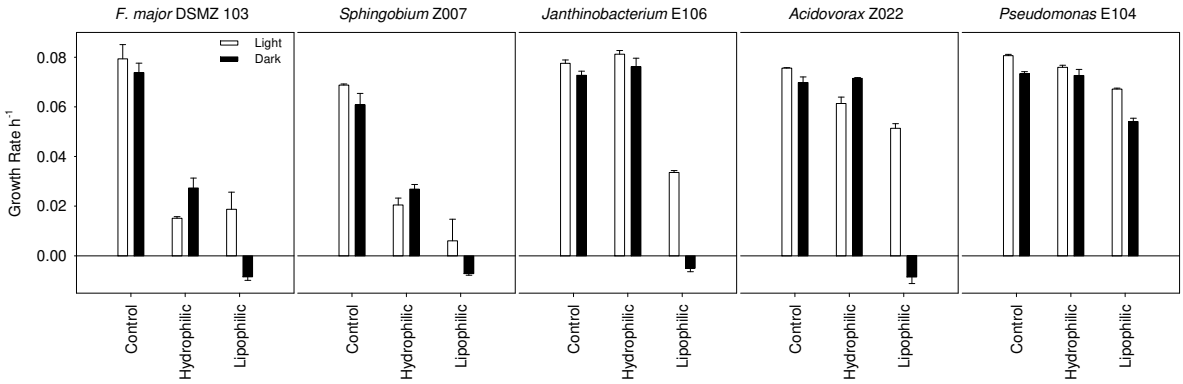


Fig. 9. Growth rates of five freshwater bacterial isolated in liquid medium amended with hydrophilic and lipophilic fractions of culture supernatants from *Poterioochromonas* sp. strain DS. White bars, supernatants from a light-adapted culture; black bars, supernatants from a dark-adapted flagellate culture. Control, growth in unamended medium. The erro bars indicate the SD of triplicates. From: **Blom & Pernthaler, 2010**.

Release of antibiotic substances

The release of antibiotic substances into the surrounding medium has been demonstrated previously for other *Ochromonas* strains (Jørgensen, 1962, Halevy*, et al.*, 1971, Hansen, 1973). Primary producers such as aquatic algae compete with bacteria for limiting nutrients, e. g. phosphate, but typically bacteria are superior competitors at low nutrient concentrations (Currie & Kalff, 1984, Mindl*, et al.*, 2005). Therefore, the excretion of antibacterial compounds might actively improve their own nutrient supply by inhibiting the growth of competing organisms **(Blom & Pernthaler, 2010)**. Moreover, the more distinct antibiotic activity of *O. danica* (the flagellate that gain more profit from autotrophy) supernatant in the agar diffusion assay would be in agreement with an anti-competitor strategy. However, supernatants of dark-adapted flagellate cultures had a more pronounced effect and acted on more strains, which can be merely the result of a higher amount of antibacterial compounds. Alternatively, other bioactive compounds might have been generated in darkness while feeding on bacteria. E.g. *P. malhamensis* was only toxic for *Daphnia ambigua* if grown hetero-, but not autotrophically (Leeper & Porter, 1995). Under natural conditions densities between 80 and 7000 ochromonads ml^{-1} have been found (Nixdorf*, et al.*, 1998, Callieri*, et al.*, 2006). Considering the dilution factor, the amount of inhibitory compounds released by the equivalent number of 500 flagellates ml^{-1} was already sufficient to reduce the growth of a susceptible bacterial strain, which clearly shows the ecological relevance of such compounds by affecting bacterial community structure under natural conditions. However, in opposite to antibiotic effects of biomass from ochromonads tested so far on pathogenic strains only (Jørgensen, 1962, Halevy*, et al.*, 1971, Hansen, 1973, Chen*, et al.*, 1994) our studies add an ecological dimension to the phenomenon already known for decades by showing growth inhibition of bacteria from various phylogenetic groups sharing the same habitat as the flagellates. Interestingly, the most sensitive strains

tested belonging to a bacterial group that exhibit inducible morphological defence strategies against flagellate predation, e.g. filamentation of *Flectobacillus* strains at high grazing pressure (Hahn*, et al.*, 1999, Corno & Jürgens, 2006, Šimek*, et al.*, 2007) or aggregation of *Sphingobium* sp. strain Z007 in the presence of *Poterioochromonas* sp. strain DS **(Blom*, et al.*, 2010b, Blom*, et al.*, 2010a)**. By contrast, the only partially affected *Janthinobacterium* sp. strain E106 and *Acidovorax* sp. strain Z022 have been shown to be moderately toxic to the flagellate in direct feeding experiments, whereas the completely unaffected *Pseudomonas* sp. strain E104 was highly toxic, and able to kill flagellate cells within minutes after addition (Blom & Pernthaler, unpublished data). While at present these are only single observations, it is conceivable that the susceptibility of aquatic bacteria to allelopathic metabolites from mixotrophic unicellular eukaryotes might be related to their specific ecological interactions.

3.4 Suboptimal light conditions negatively affect *Planktothrix rubescens* but are beneficial for accompanying *Limnohabitans* spp.

To assess the interactions between *P. rubescens* and heterotrophic bacteria in Lake Zürich, assays for bacterial community composition and incorporation of organic substrates were performed with samples from 2.5, 12.5 (*P. rubescens* maximum) and 30 m depth from Lake Zürich in late summer 2007 **(Horňák*, et al.*, 2012)**. Pre-filtered water samples from 12.5 m only were incubated in the laboratory at *in situ* temperature for 45 h under ambient light (~ 2 µmol m^{-2} s^{-1}), at 100x increased irradiance, and in the dark (roughly corresponding the natural irradiances measured at 12.5, 2.5, and 30 m depth, respectively). Additional laboratory experiments with were carried out with defined microorganisms, such as axenic *P. rubescens* strain A7 **(Blom & Jüttner, 2001)**, and *Limnohabitans parvus* and *L. planktonicus* (Kasalický*, et al.*, 2010), affiliated with the new genus *Limnohabitans*, part of the R-BT065 subcluster of *Betaproteobacteria* (Šimek*, et al.*, 2001, Hahn*, et al.*, 2010).

Abundances of all microorganisms were determined by flow cytometry, bacterial community composition and the leucine incorporation rates (as a proxy for protein synthesis) were tracked by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) (Sekar*, et al.*, 2003) combined with microautoradiograhy (MAR) (Teira*, et al.*, 2004). MAR was also applied to analyze leucine uptake by *P. rubescens* filaments. Moreover, quantitative analyses of MAR in *P. rubescens* were done using fully automated epifluorescence microscopy. Additionally, relative phycoerythrine and chlorophyll a concentrations of *P. rubescens* were measured using a fluorescence plate reader, and via inspection under an epifluorescence microscope. The mean filament length was measured by the image analysis software LUCIA D (Laboratory Imaging, Czech Republic). Total biomass

of *P. rubescens* was calculated from filament length and abundances as described in Van den Wyngaert and colleagues (Van den Wyngaert*, et al.*, 2011).

Leucine incorporation measurements of lake samples showed 14-19% active filaments in 2.5 and 12.5 m depth in lake samples, but apparently almost no activity in the hypolimnion (30 m). Moreover, active leucine uptake (of up to 20%) was also observed in the follow up incubation experiments in the laboratory. However, after 45 h of incubation at both, increased irradiation and in the dark, leucine uptake decreased to very low activity. CARD-FISH analyses of the percentages of major phylogenetic groups of bacteria in samples from three selected depths revealed a high similarity in community composition, and all groups incorporated leucine. However, whereas Betaproteobacteria (and members of the R-BT065 linage) under *in situ* conditions showed decreasing proportions of cells with active leucine incorporation along the depth profile, the relative abundances of bacteria targeted by the R-BT065 probe (*Limnohabitans* spp.) under laboratory conditions significantly increased over time, where the percentage of leucine active cells in this group was significantly elevated only in the dark incubations. Therefore, experiments with defined microbial strains were carried out with *P. rubescens, L. parvus* and *L. planktonicus*. Similarly, *L. planktonicus* reached higher abundances and leucine incorporation at ambient irradiance and in the dark treatment, but not at increased irradiance, whereas *L. parvus* only increased marginally in numbers along with a decreasing leucine uptake activity. However, both bacterial strains, and particular *L. parvus* reached higher abundances in co-culture with *P. rubescens* as compared to pure cultures only, highest abundances were found at increased irradiance **(Horňák*, et al.*, 2012)**.

Experimental manipulations clearly showed treatment-specific shift in bacterial community. Remarkably, the relative abundance of *Betaproteobacteria* doubled at increased irradiance, whereas especially the subgroup of the R-BT065 linage increased to disproportionally high abundances. These bacteria are known to rapidly respond to altering growth conditions in general (Šimek*, et al.*, 2005, Šimek*, et al.*, 2006). Interestingly, leucine uptake activity was found to be highest under dark conditions, where these bacteria additionally benefitted from the presence of high concentrations of physiologically stressed *P. rubescens* filaments. Moreover, in the co-experiments with *P. rubescens* both *Limnohabitans* strains obviously profited already only from the presence of the cyanobacterium in general (Fig. 10), whereas most significant changes were found in treatments with suboptimal light conditions for *P. rubescens*, albeit with striking differences. While total cell abundances of *L. parvus* in pure culture only increased slightly irrespective of the treatment, *L. planktonicus* reached more than one order of magnitude higher abundances at low light levels and in the darkness, which might indicate that the growth was negatively inhibited by photoinhibition as observed for bacterioplankton in the surface layers of mountain lakes (Sommaruga*, et al.*, 1997). At the same time, *L. planktonicus* much more efficiently utilized the trace levels of organic carbon available in the cultivation

medium, which might hint at a niche separation between the two species (Šimek*, et al.*, 2010).

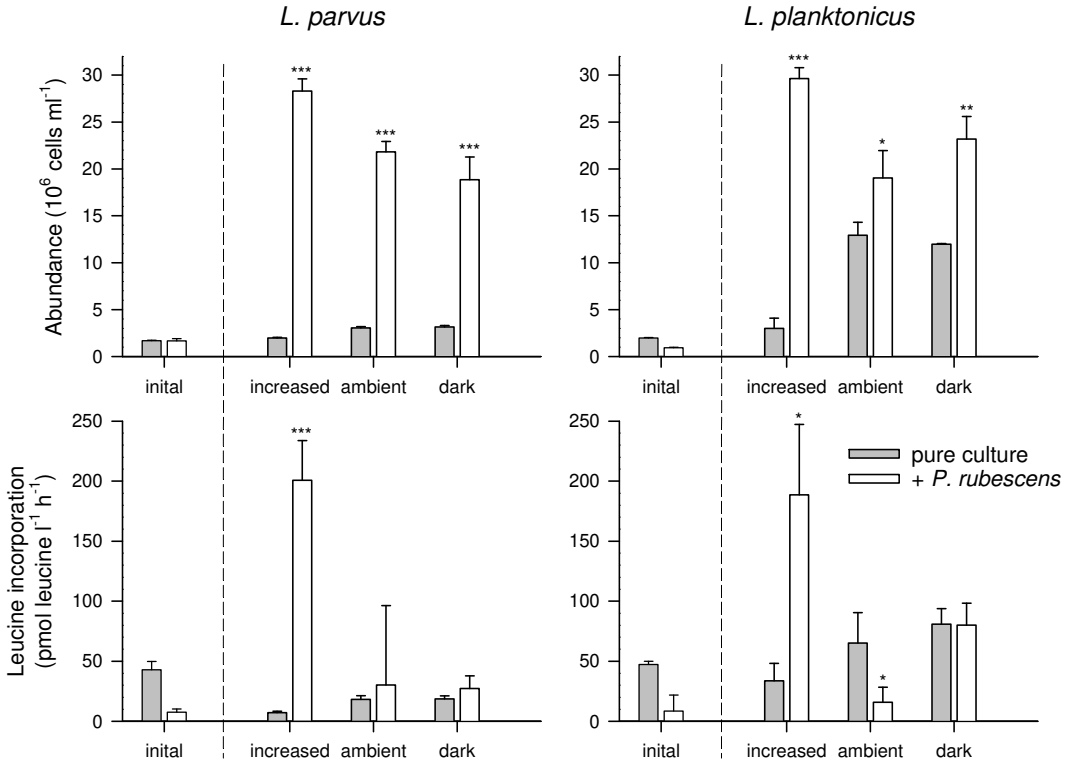


Fig. 10. Total cell numbers (upper panels) and total leucine incorporation rates (lower panels) of *L. parvus* and *L. planktonicus* in pure cultures and in co-cultures with *P. rubescens*. Initial values and data after 169 h of incubation at 3 different irradiance regimes (increased, ambient, dark) are shown. All strains were cultivated in ALW medium. Values are means of triplicates, error bars = SD. Asterisks above bars indicate that parameters in co-cultures were significantly different from pure cultures. *p < 0.05, **p < 0.01, ***p < 0.001. From: **Horňák et al., *in press***.

During this experiment *P. rubescens* significantly decreased fluorescence of both, phycoerythrine and chlorophyll *a* pigments increased light conditions, implying severe stress of this cyanobacterium. This was, moreover, accompanied by a pronounced fragmentation of *P. rubescens* filaments, as reflected in a significantly shorter mean filament length than in the other treatments at comparable total biomass. In contrast to other phytoplankton groups with high carbon exudation rates, e.g. cryptophytes (Giroldo*, et al.*, 2005), *P. rubescens* usually does not excrete a large amount of extracellular products (Feuillade*, et al.*, 1990). Even under high irradiances (accompanied by the inhibition of photosynthesis), the released carbon was shown to be always lower than 3% of the incorporated carbon (Feuillade*, et al.*, 1990). Thus, the additional bacterial growth in co-cultures at elevated light irradiation in our studies (Fig. 10) was likely due to the surplus in substrate release into the medium upon the disintegration of filaments **(Horňák*, et al.*, 2012)**. This conclusion is also supported by the substantially elevated leucine incorporation rates of both bacterial strains in this treatment only. However, at ambient light and dark conditions the leucine

incorporation for both bacterial strains in coculture with *P. rubescens* was strongly decreased, which might indicate that the cyanobacterium was less stressed, and therefore released only a low amount of organic substrates into the medium. This is supported by the strongly higher levels of phycoerythrine and chlorophyll levels of *P. rubescens* in these treatments. Moreover, the incorporation rate of leucine at ambient light conditions by *L. planktonicus* in coculture with *P. rubescens* was even significantly lower compared to the pure culture (Fig. 10), which might indicate an active excretion of allelopathic (e.g. growth inhibition) substances by *P. rubescens* into the surrounding medium to suppress bacterial growth, if the cyanobacterium is physiologically not stressed.

Therefore, it is conceivable that metabolic changes in the light-stressed *P. rubescens* along with inhibition of photosynthesis might lead to an allocation of resources. Instead of possible production and excretion of antibacterial substances to eliminate possible competitors for nutrients (as it can be seen at ambient light conditions), it is more likely, that *P. rubescens* instead sustains predominantly primary metabolism to ensure survival of cyanobacterial population. Allelopathic effects (e.g. inhibitory activity) of *Planktothrix* species have been reported so far against other cyanobacteria (Oberhaus*, et al.*, 2007) and green alga (Vasconcelos & Almeida, 2008). Thus, it is conceivable that certain *Planktothrix* species also produce allelopathic substances against heterotrophic bacteria.

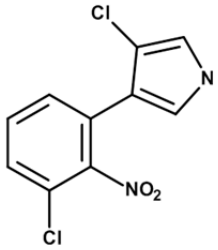
3.5 Production of the antifungal compound pyrrolnitrin in members of the *Burkholderia cepacia* complex

During last decades biological control has become more and more important in agriculture because of public concerns against the use of pesticides (Raaijmakers*, et al.*, 2002, Haas & Defago, 2005, Raaijmakers*, et al.*, 2009). Biocontrol bacterial strains such as *Bacillus*, *Streptomyces*, *Serratia*, *Pseudomonas* as well as the fungi *Fusarium* sp. were used in commercial applications (Thomashow, 1996, Berg, 2000, Cao*, et al.*, 2005) due to their potential to effectively colonize the rhizosphere of the host plants (Paulitz, 2000), and to increase their resistance against a variety of pathogens (Pieterse*, et al.*, 1996). Moreover, some of these bacteria are well known for their ability to produce antibiotics that inhibit the growth of possible plant pathogens (O'Sullivan & O'Gara, 1992). Similarly, the genus *Burkholderia* is quite often used to effectively biocontrol typical diseases caused by *Pythium* spp., *Rhizoctonia solani*, and *Fusarium* sp. (Parke & Gurian-Sherman, 2001, Coenye & Vandamme, 2003, Compant*, et al.*, 2008) via excretion of several highly effective allelochemicals (Vial*, et al.*, 2007). At least seventeen of more than fifty validly described *Burkholderia* species -so far isolated from soil, water, but also plants, insects, and fungi- belong to the so-called *Burkholderia cepacia* complex (Bcc) (Vandamme*, et al.*, 1997).

Although strains of the Bcc cluster possess a high biocontrol activity they are severely limited for use in agriculture due to their pathogenic potential to infect humans (Parke & Gurian-Sherman, 2001, Vandamme*, et al.*, 2007, Mahenthiralingam*, et al.*, 2008).

Many gram-negative bacteria are able to monitor their own population density via homoserine lactones (HSL's) in a process known as 'quorum sensing' (Fuqua*, et al.*, 1996, Whitehead*, et al.*, 2001), which enables them to coordinate and regulate certain activities such as the production of virulence factors and antimicrobial compounds, or the colonization of surfaces. Most *Burkholderia* species investigated so far communicate via N-octanoylhomoserine lactones (C8-HSL), which are synthesised by the synthase CepI. At a certain bacteria density C8-HSL binds to the LuxR-type receptor protein CepR, which then leads to either induction or repression of the target genes. *Burkholderia* species have been shown to regulate the production of extracellular proteases, chitinases, a polygalacturonase and siderophores, swarming motility and biofilm formation via the quorum sensing system (Eberl, 2006). Moreover, previous studies showed that the inactivation of *cepI* or *cepR* in *Burkholderia ambifaria* resulted in a marked reduction of its biocontrol activity, which was shown by the very low ability to inhibit fungal plant pathogens (Zhou*, et al.*, 2003). However, so for no HSL-controlled compound could be made responsible for the antifungal activity of several Bcc species and also for the molecular mechanisms behind the quorum sensing dependent control of biocontrol activity.

Antibiotic substances in Burkholderia cepacia complex



Pyrrolnitrin **(20)**

Previous work showed that the AiiA lactonase degrades HSL molecules independent of the side chain length (Dong*, et al.*, 2000), and that, therefore, the transfer of the pMLBAD-aiiA vector into *Burkholderia* strains rendered them HSL-negative (Wopperer*, et al.*, 2006). Antifungal activities of 14 *Burkholderia* strains and their AiiA-expressing derivatives were evaluated due to their inhibition zones on Malt agar against *Phytium ultimum* and *Rhizoctonia solani* **(Schmidt*, et al.*, 2009)**. Interestingly, expression of AiiA not only decreased the amount of HSL signal molecules but also stopped partially or completely antifungal activities, indicating that the AHL-dependent quorum sensing systems in *Burkholderia* species control the synthesis of antifungal agents **(Schmidt*, et al.*, 2009)**. To identify antifungal compounds in these strains, PCR assays were performed using primer pairs required for the synthesis of already known antifungal compounds such as pyoluteorin, phenazine, 2,4-diacetylsphloroglucinol and pyrrolnitrin typically found in *pseudomonads* and some other bacterial species (Dwivedi & Johri, 2003, Haas & Keel, 2003). Only the *prnD* gene, which is part of the *prnABCD* operon that directs the synthesis of pyrrolnitrin **(20)**, but no other amplicons obtained with other primer pairs, was found in some species belonging to the Bcc

complex. Subsequently, thin-layer chromatography (TLC) was used for the detection of compounds with antifungal activities in extracts of *B. lata* 383 (formerly *B. cepacia* Group K 383 (Vanlaere*, et al.*, 2008)), *B. cenocepacia* PC184, *B. ambifaria* LMG 19467, *B. pyrrocinia* LMG 21823 and *B. ubonensis* LMG 19467 and compared with synthetic pyrrolnitrin. To investigate the influence of quorum sensing on pyrrolnitrin production also the AiiA transconjugants of these strains were tested on antifungal activity. However, pyrrolnitrin production was found to be abolished or very low, indicating HSL-dependency of pyrrolnitrin production.

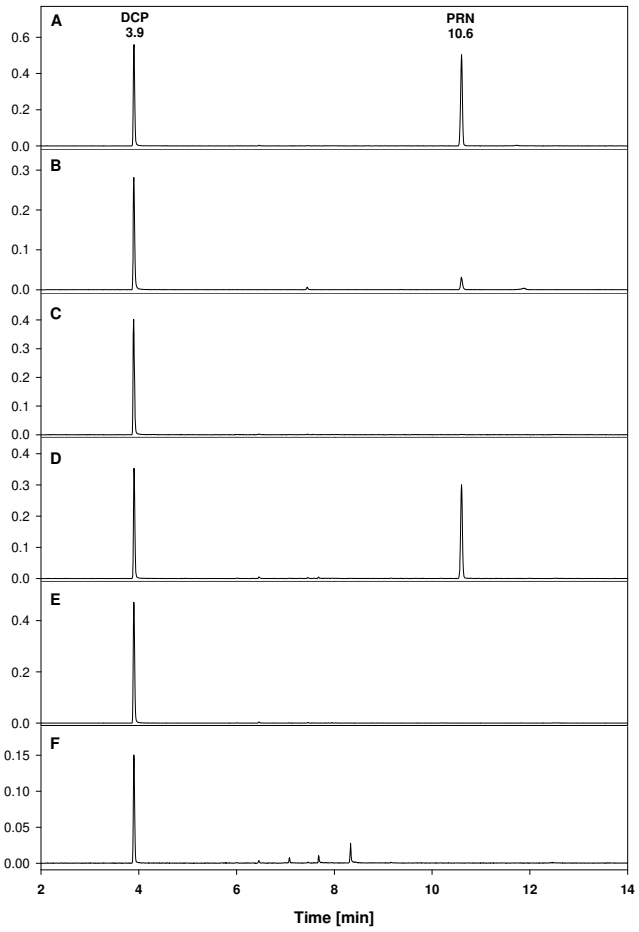


Fig. 11. GC-MS analysis of acetonitrile extracts of cultures of the wild-type *B. lata* 383 (A), the quorum-quenched derivative of 383 (B), the *cepI* mutant 383-I in the absence (C) and presence of 200 nM C8-HSL (D), the *cepR* mutant 383-R (E) and prnA mutant 383-P (F). the pyrrolnitrin standard had a retention time of 10.6 min. The fragment ion m/z = 166 was used to identify pyrrolnitrin (PRN) and dichlorophenole (DCP). From: **Schmidt et al. (2009)**.

In a next step, the genes *cepI*, *cepR* and *prnA* were inactivated in *B. lata* 383, designated as 383-I, 383-R and 383-P, respectively **(Schmidt*, et al.*, 2009)**. To clearly demonstrate that *B. lata* 383 is producing pyrrolnitrin in an AHL-dependent manner acetonitrile extracts of cultures of the wild-type 383, the AiiA-expressing transconjugant and the mutants 383-I, 383-R and 383-P were examined in an *in vitro* inhibition assay as well as by GC-MS (Fig. 11). Extracts of the wild-type strain tested against *R. solani* exhibited strong antifungal activity while extracts of the transconjugant and the quorum sensing mutants 383-I and 383-R did not show any antifungal activity. GC-MS measurements revealed only low amount of pyrrolnitrin in the extract of the transconjugant compared to wild-type strain, and no pyrrolnitrin in the 383-I, 383-R and 383-P mutants. However, addition of 200 nM C8-HSL could increase the pyrrolnitrin production again in the 383-I mutant to wild-type level. All these results unequivocally proved that the pyrrolnitrin production in *B. lata* 383 is under the control of the CepIR quorum sensing system **(Schmidt*, et al.*, 2009)**.

Previous studies have already shown that HSL-dependent quorum sensing systems in some bacteria control the production of antifungal compounds, e.g. phenazine antibiotics

produced by *Pseudomonas aurofaciens* strain 30-84, which are active against ascomycetes fungus and whose production are under the control of the PhzI/PhzR quorum sensing system (Pierson*, et al.*, 1994, Wood*, et al.*, 1997). In this study it was clearly shown that many *Burkholderia* strains of the Bcc cluster produce pyrrolnitrin in an HSL-dependent manner. It is important to keep in mind that the prnABCD operon is only present in some of the strains exhibiting antifungal activity, and that other antifungal metabolites different from pyrrolnitrin still have to be identified. However, by employing a quorum-sensing quenching approach the antifungal production was demonstrated to be HSL-regulated **(Schmidt*, et al.*, 2009)**.

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4 Infochemicals - Kairomones

4 Infochemicals - Kairomones

Chemical communication is important in aquatic environments, where visual and auditory senses are often ineffective or useless (Burks & Lodge, 2002). Thus, chemical cues mediate many aspects of interspecific relationships that determine aquatic community structure and ecosystem functioning. Chemically mediated communications has been long known not only as an important component of predator-prey systems (Dodson*, et al.*, 1994) but also for interspecific competition (Burks & Lodge, 2002), both in pelagic and benthic environments.

Current terminology and definitions of different classes of chemical cues are quite heterogeneous. Therefore, I would like to explain the use of terms in the following chapter. In general, **infochemicals** play a role in the interaction between two individuals of different species, either from the same or from another trophic level; they can be found between vertebrates, invertebrates, plants and microorganisms (Nordlund*, et al.*, 1981, Schoonhoven, 1981). Especially in aquatic systems the possibility of perception of such compounds is a clear advantage as it allows examination of the environment at night as well as in turbid water, and even visual obstacles are no obstruction (Dodson*, et al.*, 1994).

The chemoreceptors to perceive and process the information that are transmitted via infochemicals are usually classified into olfactory and gustatory chemoreceptors (Schoonhoven, 1968). Olfactory receptors are responsible for the detection of a source from a distance via odour molecules that are released into the environment. Two types of infochemicals are distinguished (Dethier, 1947, Schoonhoven, 1968). **Attractants** are infochemicals that stimulate prokaryotes as well as eukaryotes to organize their movements towards a higher concentration of infochemicals along an increasing gradient, a phenomenon also known as positive chemotaxis. Conversely, **repellents** are compounds that lead to a move away from the source (negative chemotaxis). The chemical gradients are sensed through multiple transmembrane receptors; afterwards, the signals are transmitted across the plasma membrane into the cytosol. Different intracellular signalling pathways then lead to the migratory responses (Dethier, 1947, Schoonhoven, 1968). Gustatory chemoreceptors require a direct contact between two organisms (e.g. predator and prey) to discriminate between two possible operations. Similarly as for odour compounds, **stimulants** regulate the acceptance of an operation and tend to increase activity on a certain action. Conversely, the presence of **deterrents** in an organism explains the rejection of an action or the abortion of an operation in progress, e. g. protects the prey after contact with the predator. The final response in accepting or rejecting is thought to be mediated by a balance of sensory inputs from these positive and negative chemical stimuli (Miller & Strickler, 1984, Renwick & Radke, 1987).

All the infochemicals discussed so far are produced, and stored or released by individuals of one species to affect the behaviour of a member of another species to the

benefit of the producer but not of the receiver. However, a special case within the infochemicals is the group of the so called **kairomones**. The term was originally proposed to denote substances that confer an advantage upon the receiver and a disadvantage for the emitter (Brown Jr*., et al.*, 1970). Therefore, kairomones are chemical cues that are emitted by an individual of one species to mediate interspecific interactions in a way that benefits only the individual of another species which receives it, but without benefiting the emitter, even more, being (in most cases) disadvantageous to the producer. For example, kairomones can be used to locate food (Pohnert*, et al.*, 2007). However, most kairomones produced and released by predators affect the behaviour, morphology, or life history traits (and therefore the surviving strategy) of different prey species (Dodson*, et al.*, 1994).

In many predator-prey systems studied so far the kairomones that induce the defence response of the prey species are produced during feeding (Pohnert*, et al.*, 2007, **Blom*, et al.*, 2010a**). As a consequence, the prey perceives complex chemical information, not only about type and density of the predator present but also about the prey being consumed (Larsson & Dodson, 1993). Many organisms respond upon predation or disintegrating of cells by releasing so called **alarm cues** that do not only warn members of the same species (**conspecifics**) but also heterospecifics of the presence of danger. Such cues are now known to even increase the response of the prey that was initially attributed to predator-borne kairomones only. For example, morphological changes in *Daphnia* were induced to higher extent by kairomones released from fish that were fed with *Daphnia*, but less so by fish fed with earthworms (Stabell*, et al.*, 2003). Most likely the alarm cues are not specifically produced substances, but usual constituents of the prey organisms, which are released and/or activated when wounded by the predator (Pohnert*, et al.*, 2007). However, such alarm cues only provide general information about predation risk, but not about the predator itself. Instead it seems that both - prey damage and predator digestion of conspecifics - appear to be important for the correct defence performance of the prey, although still information is lacking about the time point during the predation event at which kairomones are produced (constitutively, during prey attack or during prey digestion) (Schoeppner & Relyea, 2009). For instance, it is well known that *Daphnia* can change morphology as well as behaviour when exposed to different types of predators (*Chaoborus* and fish, respectively). For these specific responses the prey requires additional cues, besides alarm signals, that are unique to the type of predator (Laforsch & Tollrian, 2004).

However, current research has only produced limited knowledge of the chemical cues (or the bouquet of chemical cues) involved in such complex infochemically mediated communities (Pohnert*, et al.*, 2007). So far, only a few kairomones could be (partially) characterized (Dodson*, et al.*, 1994, Pohnert*, et al.*, 2007). A protein with a size of 31.5 kDa has been identified that is released by the predatory ciliate *Lembadion bullinum* and that induces morphological changes in the ciliate *Euplotes octocarinatus* (Kusch & Heckmann, 1992). The formation of cell aggregates in the green algae *Scendedesmus* and *Desmodesmus* in response to predation by different zooplankton species of fish (Hessen & Van Donk, 1993,

Lürling, 2003) is believed to be triggered by a variety of aliphatic sulphates (Yasumoto*, et al.*, 2006).

Section 4.1 Bacterial aggregate formation induced by growth state and conspecific chemical cues

Free-living bacteria in aquatic habitats typically suffer high mortality rates by grazing of flagellated protists (Pace, 1988, Pernthaler, 2005). Therefore, it is believed that bacterial aggregate or filament formation beyond edible size is an effective defence strategy against flagellate predation. Although some bacterial genotypes in freshwaters may be permanently grazing-resistant (Schauer & Hahn, 2005), others possess the possibility to partially or totally shift from consumable rod-shaped single cells to large complex morphologies, such as aggregates, flocs or microcolonies (Pernthaler*, et al.*, 1997, Šimek*, et al.*, 1997, Hahn & Höfle, 1999, Šimek*, et al.*, 1999, Salcher*, et al.*, 2005). To induce such changes two mechanisms are held responsible. For one, flagellate feeding might release surplus nutrients into the surrounding medium that might lead to morphological shifts in bacteria by providing better growth conditions (Caron*, et al.*, 1988, Verhagen & Laanbroek, 1992, Nagata, 2000). On the other hand, recent experiments provided some evidence for the action of specific chemical cues (Corno & Jürgens, 2006) in analogy with morphogenetic factors that modify other aquatic predator-prey interactions (Pohnert*, et al.*, 2007). Actually, it is conceivable that a phenotypic change towards grazing protected sizes may be constitutively triggered by high growth rates, but additionally (e.g. at suboptimal growth conditions) also by a chemical cue released by the predator. In a first step, we established a predator-prey system consisting of a freshwater bacterial isolate affiliated with *Sphingobium* sp. and the predatory flagellate *Poterioochromonas* sp. strain DS **(Blom*, et al.*, 2010a)**. We could show that both growth rate and conspecific chemical cues can induce aggregate formation in this freshwater bacterium **(Blom*, et al.*, 2010a)**.

Section 4.2 Scent of danger: Aggregation induced by supernatants from a predator-prey coculture

Reasons for the general low progress in kairomones identification are manifold and hardly to be solved. Kairomones should be very effective already in very low concentration that might be covered by side-compounds that are only active at ecologically irrelevantly high concentrations (Pohnert*, et al.*, 2007). Additionally, the availability of an appropriate bioassays to detect the effective fraction in kairomones-containing medium (von Elert & Loose, 1996) is an absolute requirement, not only for the search for kairomones, but also for a further characterization of such compounds (Tollrian & von Elert, 1994, Boriss*, et al.*, 1999). However, kairomone activity might be a result of several cues -only synergistically effective- which make bioassays an unsuitable tool to detect the effect of kairomones. One

major drawback in the study of kairomones so far was the lack of appropriate organisms and culture conditions (few individuals in high volumes to be processed) **(Blom*, et al.*, 2010b)**. Therefore, we established a model predator-prey system consisting of a freshwater bacterial isolate affiliated with *Sphingobium* sp. and the predatory flagellate *Poterioochromonas* sp. strain DS for the detection and first tentative characterization of (one or several) chemical cues that affect bacterial floc formation **(Blom*, et al.*, 2010a, Blom*, et al.*, 2010b)**.

4.1 Bacterial aggregate formation induced by growth state and conspecific chemical cues

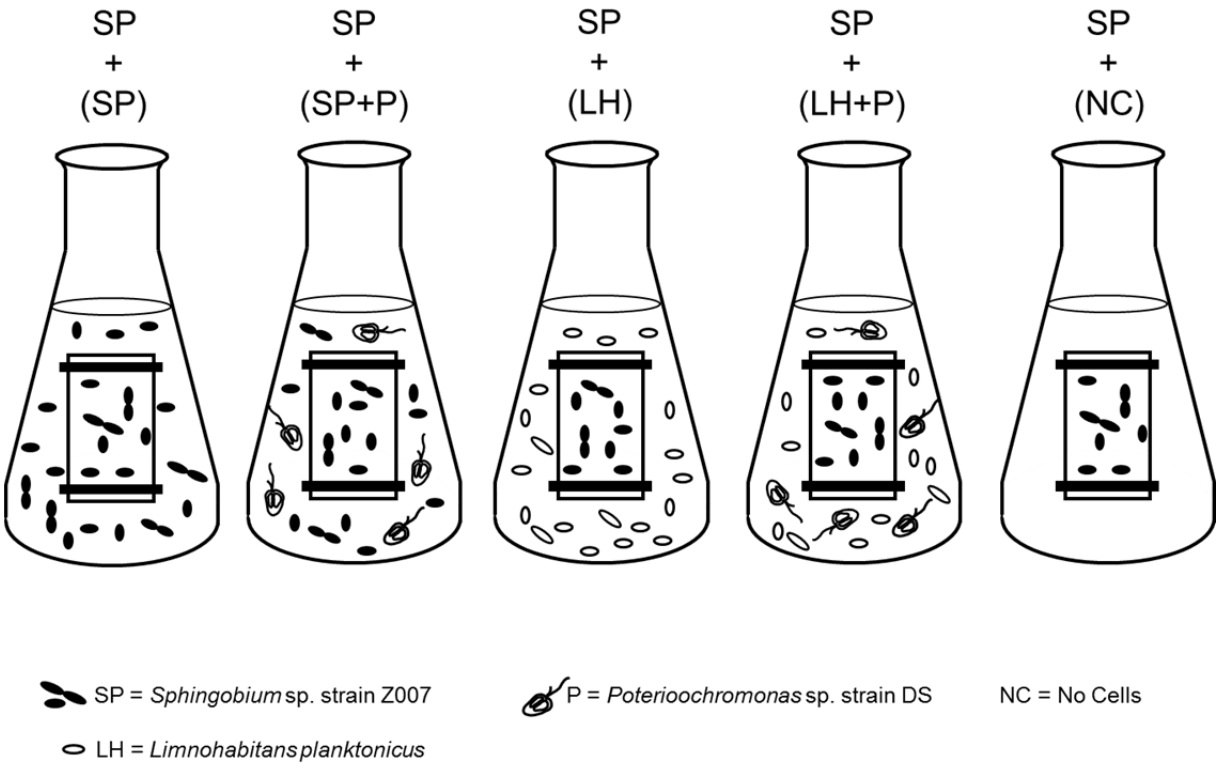

Fig. 12. Experimental setup: *Sphingobium* sp. strain Z007 inside the dialysis bags was amended with (I) *Sphingobium* sp. strain Z007 only (SP), (II) *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS (SP + P), (III) *Limnohabitans planktonicus* only (LH), and with (IV) *L. planktonicus* and *Poterioochromonas* sp. strain DS (LH + P) outside of the dialysis bags. Additionally, aggregate formation of *Sphingobium* sp. strain Z007 was studied without competing organism outside the dialysis bags (NC). According to **Blom et al., 2010a**.

Members of the *Sphingomonadaceae* family are widely distributed across freshwater habitats, densities of up to $<10^6$ ml^{-1} are quite common (Piccini*, et al.*, 2006). Moreover, relatives of this family have been frequently found in newly as well as aged freshwater organic aggregates (so called *lake snow*) (Knoll*, et al.*, 2001, Schweitzer*, et al.*, 2001); additionally, co-aggregation with other species was also observed in some species (Min &

Rickard, 2009). Therefore, members of the *Sphingomonadaceae* seemed to be appropriate organisms for characterizing the aggregate formation phenomenon.

Sphingobium sp. strain Z007 was first isolated from the surface water layer of mesotrophic Lake Zürich in 2006 **(Blom & Pernthaler, 2010)**. This strain forms cell aggregates in pure culture but also appears to phenotypically respond to predators during batch growth. Axenic cultures of the facultatively mixotrophic flagellate predator *Poteriochomonas* sp. strain DS were produced as described elsewhere **(Blom & Pernthaler, 2010)**. Aggregate formation of *Sphingobium* sp. strain Z007 was studied during different growth phases with and without predation, and keeping bacteria spatially separated from predators (Fig. 12). In addition, a second bacterial strain *L. planktonicus* strain II-D5 (Kasalický*, et al.*, 2010) with no morpholocial plasticity was used either as competitor or as alternative prey in order to assess if cell aggregation in *Sphingobium* sp. strain Z007 was also affected by interspecific interactions. In a last treatment aggregate formation was studied without competing organism outside (Fig. 12).

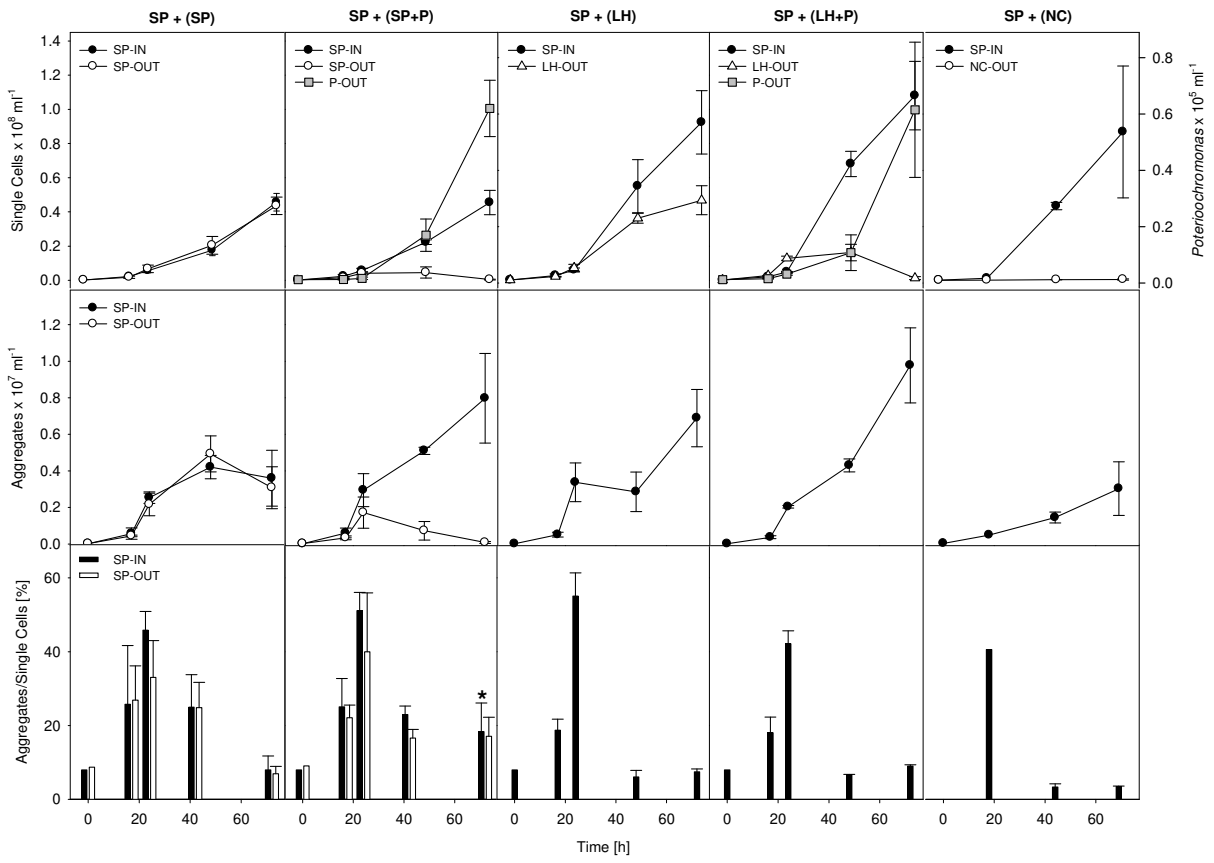

Fig. 13. Changes in the abundance of single cells of *Sphingobium* sp. strain Z007 (SP), *Limnohabitans planktonicus* (LH), and *Poterioochromonas* sp. strain DS (P) (upper panels); changes in aggregate numbers (middle panels); changes of the ratios of aggregates to single cells (lower panels) of *Sphingobium* sp. strain Z007 (SP) inside (IN) and outside (OUT) of the dialysis bags. Asterisk above bar indicates that the ratio of aggregates to single cells inside the bags after 70 h was significantly different from the control ($p < 0.05$; tested by ANOVA, followed by Dunnett`s post hoc tests). From: **Blom et al., 2010a**.

Sphingobium sp. strain Z007 in pure culture showed highest proportions of cell aggregates during the early exponential growth phase (Fig. 13), whereas the population shifted towards single cells during the later growth stage. This suggests that aggregate formation in this strain is enhanced during optimal growth conditions. This phenomenon is already well known. For example, *Azospirillum brasiliensis* Sp7 formed the highest fractions of aggregates during the early exponential growth phase (Nikitina*, et al.*, 2001) and it was shown similarly for *Pseudomonas aeruginosa* biofilms, which rapidly dispersed and released single cells into the medium under suboptimal growth conditions (Schleheck*, et al.*, 2009). This might be explained as indirect anti-predation strategy (Hahn et al., 1999). The removal of competing bacteria and the release of additional nutrients as a result of flagellate grazing at the same time might allow for faster growth of grazing-resistant bacteria (Šimek*, et al.*, 2007). Additionally, the aggregate formation at early growth stages could be a beneficial strategy for planktonic bacteria for exploiting sources of organic matter in the water column, which are steepest, e.g. in the phycosphere of senescent algal cells (Grossart & Simon, 1998), in an otherwise inhomogeneously distributed environment. However, at the same time there are also metabolic and ecological disadvantages of such growth strategy, e.g. vulnerability to larger predators (Jürgens & Güde, 1994, Hahn*, et al.*, 2000). Interestingly, the overall densities of *Sphingobium* sp. strain Z007 inside the dialysis bags in our study were higher in the presence of *L. planktonicus*. Of course, it is conceivable that the two bacterial strains did not entirely overlap in their respective substrate spectra. Alternatively, a more active, e.g. auxotrophic relationship might be assumed, in which *Sphingobium* sp. strain Z007 profited from additional substrates released from *L. planktonicus*.

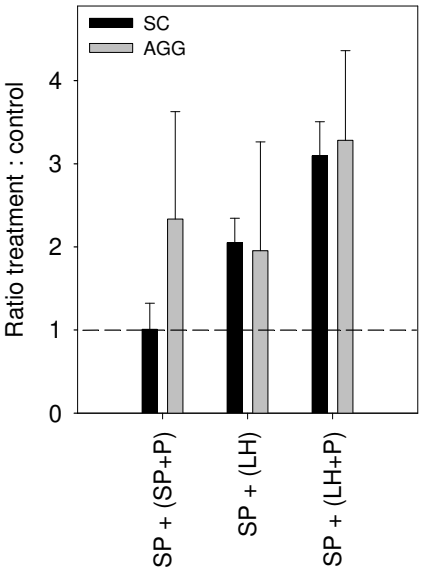

Fig. 14. Ratio of the mean abundances of single cells (SC, black columns) and aggregates (AGG, grey columns) of *Sphingobium* sp. strain Z007 in the different treatments to the corresponding values in the control treatment (SP) at the last sampling time point. In this figure, ratios of two experiments with identical setup were merged together.

However, our results go beyond providing evidence for growth-related cell aggregation in *Sphingobium* sp. strain Z007. While the proportions of aggregates inside the dialysis bags decreased in four out of five treatments, a significantly higher fraction of aggregates was only found, when flagellates were allowed to feed on *Sphingobium* sp. strain Z007 outside the dialysis bags at comparable growth rates in all treatments. At the same time, the final abundances of single cells formed inside the bags were identical irrespective of whether these bacteria were grazed upon or not. The effect of flagellate predation and a competing bacterial strain can be assessed by comparing the ratio of the abundances of single cells and aggregates inside the dialysis bags in various treatments with the corresponding values in the control treatment (Fig. 14). Thus, the higher ratio of aggregates in the SP+P treatment at

the end of the experiment was not a consequence of predator-induced nutrient recycling, but of a chemical stimulus. Moreover, the involved factor appeared to be conspecific, because cell aggregation was only triggered upon feeding on *Sphingobium* sp. strain Z007, but not *L. planktonicus*. These findings clearly extended earlier reports of cell filamentation induced by chemical cues (Corno & Jürgens, 2006). It is interesting to note that smaller aggregates were not protected from being grazed outside the dialysis bags. This might indicate that this kind of defence strategy is a rather general response and might be more or less effective for different flagellate species.

4.2 Scent of danger: Aggregation induced by supernatants from a predator-prey coculture

The exact nature and molecular action of most kairomones are still unknown, also because of the difficulty to establish an appropriate bioassay to rapidly detect the effective fraction and to further characterize such compounds (Pohnert*, et al.*, 2007). The most commonly used approach is called bioassay-guided fractionation. Water samples, containing the bioactive compound(s) or enriched extracts are separated using analytical techniques (Miralto*, et al.*, 1999, **Blom & Jüttner, 200**1, Pohnert*, et al.*, 2007). The bioactivity is stepwise verified using a bioassay. However, most of the so far studied predator-prey systems are rather inconvenient for identification of a chemical factor. Depending on the organisms and the therefore resulting vessel sizes or basins used, some bioassays will yield relatively large volumes to be processed, and a single experiment might last for 10 to 20 days (Corno & Jürgens, 2006, Blom*, et al.*, 2010b). In contrast, a bacterial bioassay based on static batch cultures would be experimentally simple and rapid, it could be set up in a highly parallel manner in small volumes, and a high number of organisms can be tested that strengthen statistically analyses **(Blom*, et al.*, 2010b)**. However, neither the appropriate bacteria nor the conditions for a batch culture model of chemically induced morphological grazing resistance were available so far. However, aggregate-forming bacteria seemed to be a promising target in the search for a

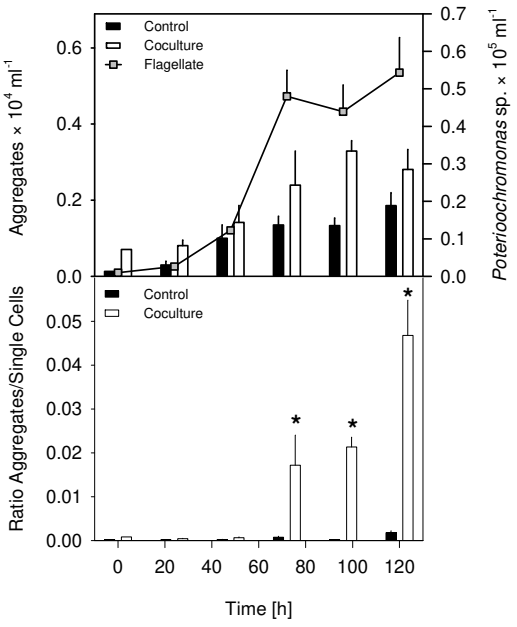

Fig. 15. Time course of changes in abundance of *Poterioochromonas* sp. strain DS and of aggregates in control (bacteria only) and in coculture with the flagellate during batch culture incubation in artificial lake water medium (upper panel). Ratio of aggregates to single cells of Sphingobium sp strain Z007 in controls and in coculture. Error bars indicate the standard errors of three replicates. Asterisks, significantly higher than control treatments at P < 0.01. Taken from **Blom et al., 2010b**; modified.

kairomones. We improved the batch culture bioassay consisting of the freshwater bacterium *Sphingobium* sp. strain Z007 and the flagellate predator *Poterioochromonas* sp. strain DS for the detection and first tentative characterization of one or several chemical factors that would affect bacterial aggregation in a model predator-prey system **(Blom*, et al.*, 2010b)**.

Inoculation of *Poterioochromonas* sp. strain DS into batch cultures of *Sphingobium* (in oligotrophic artificial lake water, Fig. 15) resulted in an increase in the aggregated bacterial subpopulation paralleled by a significant higher ratio of aggregates with a larger size than flagellates. Likewise, increased formation of suspended microcolonies by *Pseudomonas* sp. strain MWH1 (Hahn*, et al.*, 2000) and by two strains of the *Betaproteobacteria* class (Hahn*, et al.*, 2004) were found in static batch cultures.

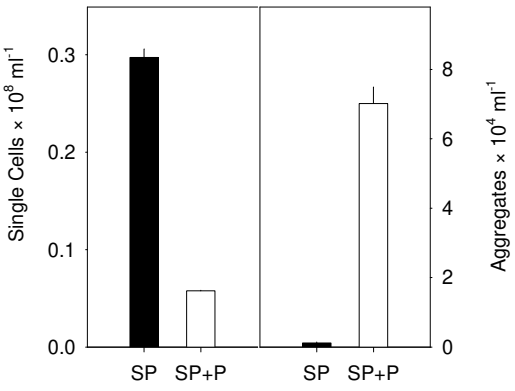

Fig. 16. Abundance of single cells (left panel) and aggregates (right panel) of *Sphingobium* sp. strain Z007 in the supernatants of pure bacterial cultures (black bars; SP) and in supernatants of cocultures with *Poterioochromonas* sp. strain DS (white bars; SP+P). Error bars indicate the standard errors of three replicates.

An important step for the improvement of the bioassay was to produce supernatants that were entirely free of flagellates. As filtration procedures (as applied in the past, (Hahn*, et al.*, 2000, Corno, 2006)) might lead to false positive- or negative results (Lürling & Beekman, 2002), a combination of three gentle centrifugations steps followed by deep freezing of the supernatant seemed to be more suitable to avoid introduction of any additional components **(Blom*, et al.*, 2010b)**. Dilution of the supernatants (as done before (Hahn*, et al.*, 2000, Corno, 2006, Corno & Jürgens, 2006)) was additionally avoided, as it might decrease the concentration of trigger compounds. Several media were tested, but the best results in aggregate formation in the supernatants were found in rich medium (Fig. 16). Therefore, this

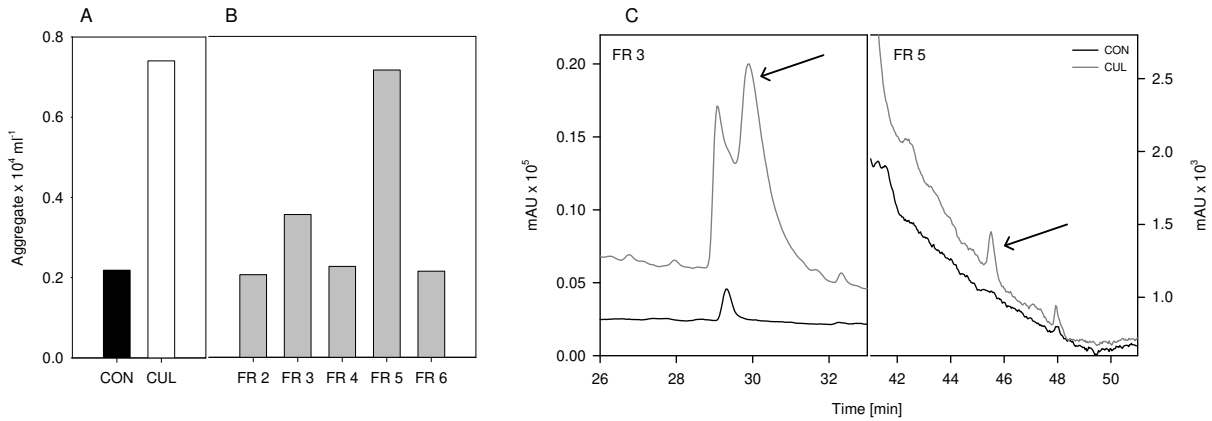

Fig. 17. Number of aggregates of *Sphingobium* sp strain Z007 after 48h of incubation amended with 50% aq. methanolic fractions of Control (CON) and Coculture (CUL) obtained by C18 cartridge separation (A). Subfractions obtained by HPLC separations of the 50% aq. methanolic fraction obtained from the coculture (B). HPLC chromatograms of the two 50% aq. methanolic fractions at 254 nm (C).

medium was chosen for all follow-up experiments of the bioassay-guided fraction procedure.

Supernatants of bacterial monocultures and of cocultures of bacteria and flagellates could be successfully fractionated using a C18 cartridge. Only upon addition of the 50% aqueous methanolic extract of the coculture, the aggregate formation was significantly higher than in all other fractions tested (Fig. 17A). Therefore, this fraction was used for further investigations using common reversed-phase HPLC methods. Interestingly, the subfractions obtained by this procedure could again be proved to disproportionally induce cell aggregation **(Blom*, et al.*, 2010a, Blom*, et al.*, 2010b)**. Further investigations gave evidence for at least two subfractions with a positive effect on aggregate formation in *Sphingobium* sp. strain Z007 (Fig. 17B). Moreover, aggregate formation comparable with the success of the 50% aq. methanolic fraction obtained from the supernatant of the coculture was demonstrated for the first time in fraction 5 (unpublished data). Interestingly, fraction 5 produced many but small aggregates, whereas fraction 3 led to rather big sized aggregates. Comparisons between the chromatograms (at 254 nm) of both 50% aq. methanolic fractions (obtained from pure bacterial culture and coculture with flagellates) indicated the presence of additional compounds in the fraction obtained from the coculture (Fig. 17C, arrows). Thus, aggregate formation in *Sphingobium* sp. strain Z007 possibly takes place in two steps with the help of (at least) two different compounds.

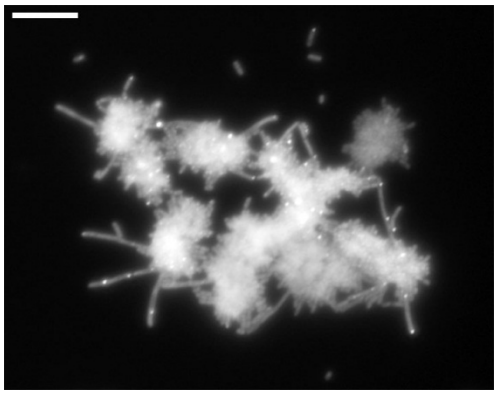

Fig. 18. Micrograph of aggregates from a coculture of *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS after sorting and DAPI staining. Bar = 10 µm.

This is supported by the fact that aggregates of *Sphingobium* sp. strain Z007 show typically two different components: long filamentous cells and small coccoid to rod-shaped cells, which are arranged around the middle part of the aggregates (Fig. 18). Therefore, aggregate formation might be explained in the following way: after first filamentation of few cells (possibly triggered by one compound) a subsequent enlargement of the aggregates takes place due to the clustering of many smaller cells (likely triggered by a second compound). It is interestingly to note that the unknown compound in fraction 3 exhibited the typical absorption spectrum of a nucleoside (unpublished data), some of which are known to arrest effectively cell growth and thus decreasing cell division (Carvalhal*, et al.*, 2003).

4.3 Outlook: Evolution of grazing resistance

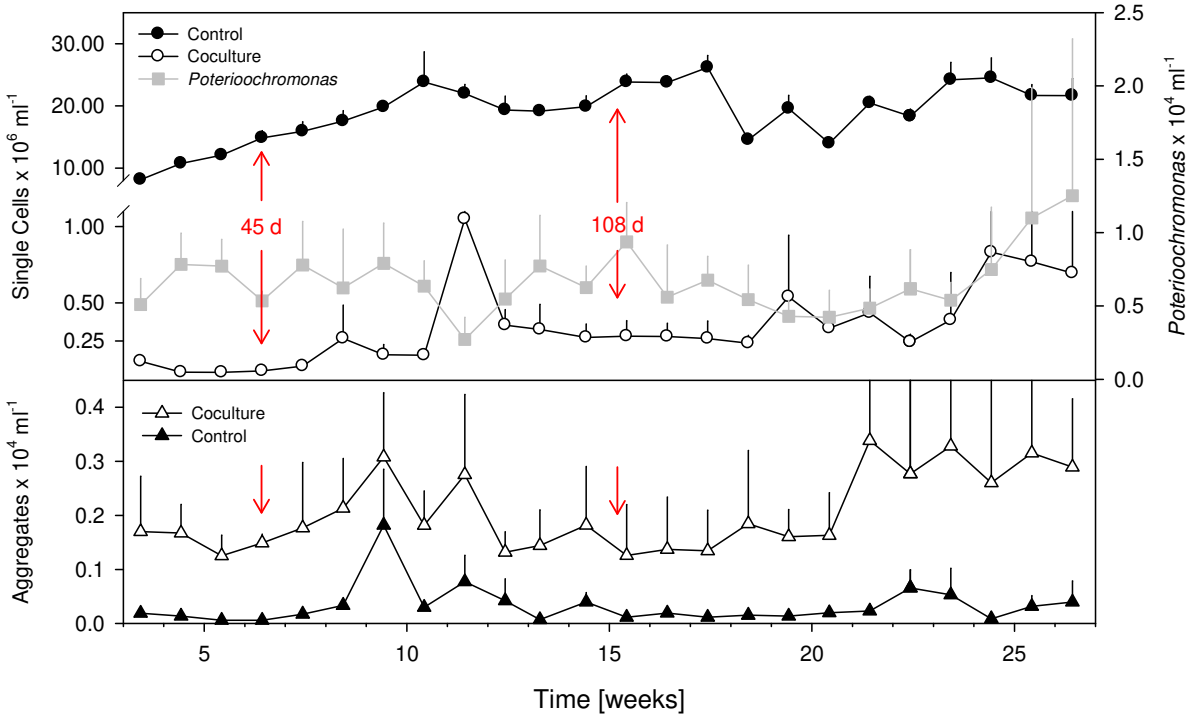

Fig. 19. Number of single cells (upper panel) and aggregates (lower panel) of *Sphingobium* sp. strain Z007 in pure bacterial culture (black) and in coculture (white) with *Poterioochromonas* sp. strain DS (grey area).

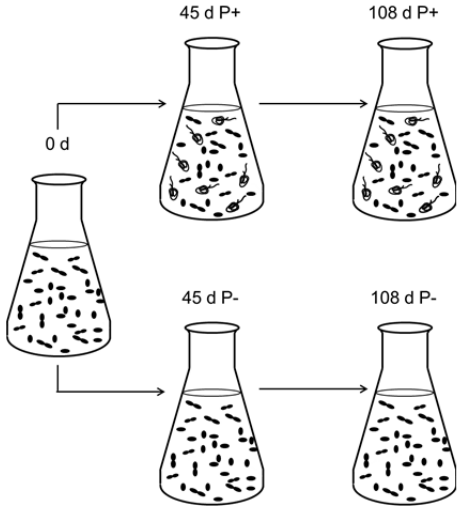

Fig. 20. Experimental setup. *Sphingobium*, at time point 'zero' (0 d), evolved with (P+) or without predators (P-) at two different time points (45 d; 108 d).

The size-selective nature of protistan grazing is an important factor that not only shifts the microbial community structure towards protected species but also might select for specific adaptations in bacteria. Under strong grazing pressure bacterial traits that increase the ability to escape predation should be favoured by natural selection. Therefore, new bacterial prey genotypes with phenotypes of increased antipredator fitness must continuously evolve. To assess evolutionary changes of the above described *Sphingobium* strain in response to predation we set up long-term experiments with pure bacterial cultures and co-cultures consisting of the bacterium and the flagellate predator. Bacterial populations were allowed to evolve for several months by serial propagation in oligotrophic medium.

The experiments were run in 300 ml Erlenmeyer flasks in a final volume of 100 ml at 18°C in the dark. Five batch cultures were inoculated with *Sphingobium* sp. strain Z007 and *Poterioochromonas* sp. strain DS in natural concentrations and ratios. As controls, five batch cultures were inoculated with *Sphingobium* sp. strain Z007 only. Once per week 10 ml of the treatments were transferred into 90 ml fresh oligotrophic medium. Subsamples were taken three times per week (72, 120, and 168 h after inoculation into fresh medium), fixed with glutaraldehyde, and subsequently analysed by flow cytometry. After an adjustment period of approximately 10 weeks characterized by a continuous increase in single cell numbers, the control treatment stayed more or less stable with single cell numbers around 2.1 x 10^7 ml^{-1} (Fig. 19). In the coculture, the number of single cells first decreased to a minimal concentration of 50'000 cells ml^{-1}. However, the number of single cells increased again to mean numbers around 0.45 x 10^6 ml^{-1} after approximately 10 weeks. *Poterioochromonas* sp. strain DS could keep a stable population around 6'500 flagellates ml^{-1} (Fig. 19). The increase in single cells in both treatments within the first 10 weeks might indicate a substrate adaptation of *Sphingobium* sp. strain Z007. During all the time, the number of aggregates in both treatments stayed more or less stable, around 350 ml^{-1} in the control treatment and 2100 ml^{-1} in the coculture treatment (Fig. 19). Every two to three weeks, bacterial strains were isolated on agar plates (nutrient rich medium), and were stored afterwards at -80°C until further analysis.

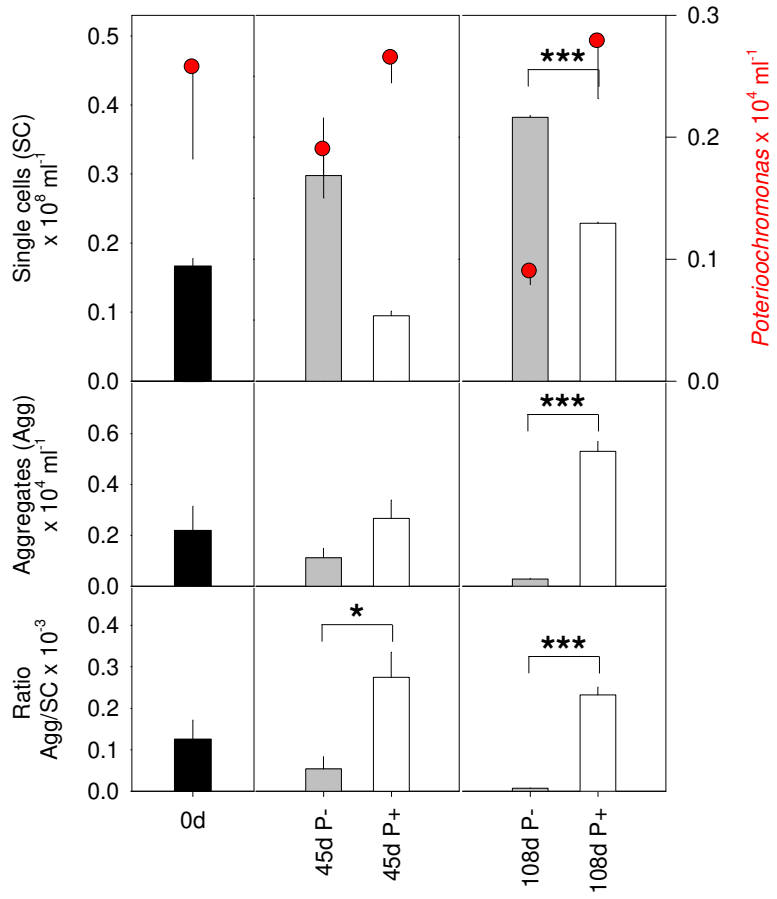

Fig. 21. Single cells (upper panel), aggregates (middle panel), and ratio of aggregates to single cells (lower panel) of *Sphingobium* sp. strain Z007 under grazing pressure of *Poterioochromonas* sp. strain DS (red circles) and from three different time points, either evolved with (P+) or without predators (P-).

Growth and phenotypic shifts of adapted *Sphingobium* strains were compared at three different time points (0 d, 45 d, and 108 d), evolved either with (P+) or without *Poterioochromonas* (P-) (Fig. 20). Bacteria from all three time points were first tested in

direct contact experiments **(Blom*, et al.*, 2010b)** in nutrient rich medium, with the addition of *Poterioochromonas* sp. strain DS. The single cell numbers at 108 d (both P- and P+) were higher compared to all other treatments, as already observed in the long term experiment (Fig. 21). However, in both treatments derived from P+ treatments (45 d and 108 d), the numbers of single cells were lower compared to the corresponding P- treatments. This lower amount of single cells numbers was accompanied by generally higher amount of aggregates, and thus resulting in higher aggregate to single cells ratios as compared to P- treatments. Significant differences between both treatments were already measurable after approximately 160 generations (45 d), but were more pronounced at 108 d.

Using populations of fast-replicating microorganisms *in vitro* to study experimental evolution in "real time" is nowadays a very common approach (Buckling*, et al.*, 2009). However, so far mainly the coevolution of bacteria and their corresponding phages have been used in order to study bacterial adaptability to novel environmental conditions (Pal*, et al.*, 2007, Buckling*, et al.*, 2009). For instance, after fewer than 200 bacterial generations about 25% of the populations coevolved with phages showed 10-100-fold increases in mutation rates, compared to no observed changes in mutation rate in populations evolved in the absence of phages (Pal*, et al.*, 2007). Thus, bacteriophages may play an important role in the evolution of bacterial mutation rates (Pal*, et al.*, 2007). We used bacteria and their common predators, flagellates, in our coevolution experiment. Interestingly, also in our approach relatively short intervals of about 160 bacterial generations (45 d) already resulted in visible differences of bacterial growth and aggregation behaviour between strains from the two cultivation conditions (with and without predators). Gene sequence analyses to study possible mutation rates and the genetic diversity of the evolved *Sphingobium* sp. Z007 strains are still lacking. Moreover, the presence of conspecific chemical cues in the coculture treatment and the possible amplified effects of these infochemicals on P+ evolved strains compared to P- bacterial strains have to be carefully examined. However, already this first insight into coevolution studies with bacteria and their predators point at the importance of flagellate predation as another important driver of evolutionary changes in aquatic bacteria, and at the possible evolution of grazing resistance under the conditions applied.

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Research Mission

Ecological understanding of the origin, the presence, and the function of bioactive compounds that mediate interactions within and between living aquatic microorganisms.

Education

1998-2003	**Dissertation**, Limnological Station, University of Zürich (UZH), Switzerland Dr. sc. nat., awarded 11/2003
1992-1998	**Diploma**, Department of Animal Physiology, University of Münster, Germany Dipl. Biol., awarded 04/1998
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Research

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2003-2006	**Postdoctoral Fellow** (UZH / Research Advisor: Prof. Dr. F. Jüttner)
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1998	**Internship** (University of Münster / Advisor: Prof. Dr. W. Stöcker)
1997-1998	**Dipl. Biol.** (University of Münster / Advisor: Prof. Dr. R.J. Paul)

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Reference-No. SNF PDFMP3_132466/1

01/2012-12/2015 ESSEM COST Action ES1105

European Cooperation in the field of Scientific and Technical Research (COST). ***Proposal participant; Management Committee member, Working group member (49 participants from 23 European Countries;*** **Proposer: Dr. Triantafyllos Kaloudis (Athens Water Supply and Sewerage Company)**

Title: 'Cyanobacterial blooms and toxins in water resources: Occurrence, impacts and management'.

Reference-No. Oc-2011-1-9346

Scientific communications

16 Publications in international peer reviewed journals, **1 patent** in collaboration with Prof. Dr. K. Gademann (University of Basel, Switzerland), and **1 book chapter** as main author (Protocols on Cyanobacterial and Algal Research). Articles have been published in journals covering the fields of Organic Chemistry, Natural Products, Environmental Sciences, Environmental Microbiology, and Toxicology. Times cited in Web of Science (as of Feb, 26th 2012): 259; h-index: 10

Highlights of own scientific work by others

Article #10 highlighted on www.chemistry.org (2006, March 27th)

Article #14 selected as "hot article" by the American Chemical Society (Environmental Month Feature)

Altogether 14 oral presentations; 6 invited lectures (at universities and international student courses across Europe); 8 oral presentations at international congresses and symposia, e.g. SIL (Societas Internationalis Limnologicae), Conference on Toxic Cyanobacteria, and Symposium on Aquatic Microbial Ecology (SAME), Symposium on Current Issues and Perspectives in Aquatic Microbiology and Microbial Ecology; 3 poster presentations at international congresses (ASLO meeting; Congress of Toxicology; SAME)

Selected Extramural Service

National Contact of Switzerland, UNESCO-Project CYANONET (IHP-Programme, 2005)

Reviewer for multidisciplinary peer-reviewed journals, such as PLoS ONE, Aquatic Ecology, International Review of Hydrobiology, Journal of Phycology, Microbial Ecology, The Scientific World Journal

Professional Memberships

Member of the Society of International Limnologists (SIL), and of the Centre for Xenobiotic and Environmental Risk Research (XERR).

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Prof. Dr. Karl Gademann	Department of Chemistry, University of Basel, Switzerland
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Teaching Experience

A) Supervision (and Co-supervision) of Diploma- and PhD theses

Supervisor of 6 Diploma/Master and 1 Doctorate (PhD) theses. Theses are written in English, most results are published in peer-reviewed journals (or are currently in preparation).

Diploma students:

- Julien Beuchat, finished 2003: Structure and Function of Bioactive Peptides from *Oscillatoria agardhii* NIVA 18 and other Freshwater Bacteria; University of Zürich, Diploma-Thesis, 60 pp.
- Michael Zeder 2005: Cyclic Peptides in Cyanobacteria and their effects on Apoptosis Enzymes of Eukaryotes; University of Zürich, Diploma-Thesis, 54 pp.

Master students:

- Reto Bertschinger, finished 2008: Trophic Interactions Between the Mixotrophic Flagellate *Poterioochromonas* sp. and Freshwater Bacterial Isolates; University of Zürich, Master-Thesis, 85 pp.
- Thomas Ammann, finished 2008: Induction of Bacterial Defense Mechanisms by the Mixotrophic Flagellate *Poterioochromonas* sp.; University of Zürich, Master-Thesis, 93 pp.
- Yannick Zimmermann, finished 2009: Chemically Induced Aggregate Formation of Freshwater Bacteria in the Presence of a Flagellate Predator; University of Zürich, Master-Thesis, 59 pp.
- Michael Baumgartner, finished 2012: Aggregate formation and evolutionary adaptation to predation in *Sphingobium* sp. isolates; University of Zürich, Master-Thesis, 69 pp.

Current Supervision Activities (started January 2011)

PhD-Students:

- Esther Kohler: Hypothetical Functional Equivalents of Microcystins (finishing December 2013).

B) Teaching Activities (Lectures and Courses)

Co-Supervision (1999-2011) **and Administration** (2002-2006) of more than 20 student courses both realized by the University Zürich (**UZH**), and Eidgenössische Technische Hochschule Zürich (**ETHZ**) (together with various other co-supervisors)

SS 99;00;01;02	2367 Blockkurs in Limnologie II
WS 99/00;00/01;01/02	2454 Blockkurs in Limnologie I
WS 02/03	2603 Blockkurs Limnologie
SS 2003	2733 Blockkurs Biochemische Limnologie
WS 03/04	2787 Blockkurs Limnologie
SS 2004	2833 Blockkurs Biochemische Limnologie
WS 04/05	551-1107-00 Praktikum Experimentelle Mikrobiologie I (ETHZ)
WS 04/05;05/06	BIO 281 Limnologie (Mikrobiologie)
SS 2005	BIO 290 Biochemische Limnologie
WS 06/07; HS 07	BIO 281 Aquatische Mikrobiologie
SS 07; FS 08/09/10/11	BIO 290 Aquatische mikrobielle Ökologie
SS 2010	International Student Course, SItE*MICRO*, Rome, Italy;

Co-Lecturer at the UZH and ETHZ in the field of Limnology, Microbiology, Secondary Metabolites, and Aquatic Chemical Ecology (since 2004, together with various other lecturers)

SS 2004	Biochemische Limnologie
WS 04/05	BIO 281 Limnologie (Mikrobiologie)
WS 04/05	551-0709-00 Limnologie (ETHZ)
SS 2005	BIO 290 Biochemische Limnologie
SS 2005	Biologie IV (Grundstudium, Vertretung)
WS 05/06	BIO 281 Limnologie (Mikrobiologie)
SS 07; FS 08/09/10/11	BIO 290 Aquatische mikrobielle Ökologie
FS 2008	Ökologie und Biodiversität, Biologie und Gesellschaft (Grundstudium, Vertretung)
FS 2010	BIO 308 Einführung in die Limnologie
FS 2011	BIO 308 Introduction to Limnology (Inland water ecosystems)

SS = summer semester; WS = winter semester: HS = autumn Semester; FS = spring semester

List of Publications

Judith Blom

A) Peer reviewed journals

1. Horňák, K., M. Zeder, J.F. Blom, T. Posch and J. Pernthaler, Suboptimal light conditions negatively affect the heterotrophy of *Planktothrix rubescens* but are beneficial for accompanying *Limnohabitans* spp. Environmental Microbiology, **14**:765-778.

2. Blom, J.F., Y.S. Zimmermann, T. Ammann and J. Pernthaler, Scent of danger: floc formation by a freshwater bacterium is induced by supernatants from a predator-prey coculture. Applied and Environmental Microbiology, 2010. **76**: 6156-6163.

3. Blom, J.F. and J. Pernthaler, Antibiotic effects of three strains of chrysophytes (*Ochromonas*, *Poterioochromonas*) on freshwater bacterial isolates. Fems Microbiology Ecology, 2010. **71**: 281-290.

4. Blom, J.F., K. Horňák, K. Šimek and J. Pernthaler, Aggregate formation in a freshwater bacterial strain induced by growth state and conspecific chemical cues. Environmental Microbiology, 2010. **12**: 2486-2495.

5. Gademann, K., C. Portmann, J.F. Blom, M. Zeder and F. Jüttner, Multiple toxin production in the cyanobacterium *Microcystis*: isolation of the toxic protease inhibitor cyanopeptolin 1020. Journal of Natural Products, 2010. **73**: 980-984.

6. Schmidt, S., J.F. Blom, J. Pernthaler, G. Berg, A. Baldwin, E. Mahenthiralingam and L. Eberl, Production of the antifungal compound pyrrolnitrin is quorum sensing-regulated in members of the Burkholderia cepacia complex. Environmental Microbiology, 2009. **11**: 1422-1437.

7. Portmann, C., J.F. Blom, M. Kaiser, R. Brun, F. Jüttner and K. Gademann, Isolation of aerucyclamides C and D and structure revision of microcyclamide 7806A: heterocyclic ribosomal peptides from *Microcystis aeruginosa* PCC 7806 and their antiparasite evaluation. Journal of Natural Products, 2008. **71**: 1891-1896.

8. Portmann, C., J.F. Blom, K. Gademann and F. Jüttner, Aerucyclamides A and B: Isolation and synthesis of toxic ribosomal heterocyclic peptides from the cyanobacterium *Microcystis aeruginosa* PCC 7806. Journal of Natural Products, 2008. **71**: 1193-1196.

9. Christiansen, G., W.Y. Yoshida, J.F. Blom, C. Portmann, K. Gademann, T. Hemscheidt and R. Kurmayer, Isolation and structure determination of two microcystins and sequence comparison of the McyABC adenylation domains in *Planktothrix* species. Journal of Natural Products, 2008. **71**: 1881-1886.

10. Hoeger, S.J., D. Schmid, J.F. Blom, B. Ernst and D.R. Dietrich, Analytical and functional characterization of microcystins [Asp^3]MC-RR and [D-Asp^3, Dhb^7]MC-RR: Consequences for risk assessment? Environmental Science & Technology, 2007. **41**: 2609-2616.

11. Blom, J.F., T. Brutsch, D. Barbaras, Y. Bethuel, H.H. Locher, C. Hubschwerlen and K. Gademann, Potent algicides based on the cyanobacterial alkaloid nostocarboline. Organic Letters, 2006. **8**: 737-740.

12. Blom, J.F., H.I. Baumann, G.A. Codd and F. Jüttner, Sensitivity and adaptation of aquatic organisms to oscillapeptin J and [D-Asp^3, Dhb^7]microcystin-RR. Archiv Fur Hydrobiologie, 2006. **167**: 547-559.

13. von Elert, E., L. Oberer, P. Merkel, T. Huhn and J.F. Blom, Cyanopeptolin 954, a chlorine-containing chymotrypsin inhibitor of *Microcystis aeruginosa* NIVA Cya 43. Journal of Natural Products, 2005. **68**: 1324-1327.

14. Blom, J.F. and F. Jüttner, High crustacean toxicity of microcystin congeners does not correlate with high protein phosphatase inhibitory activity. Toxicon, 2005. **46**: 465-470.

15. Blom, J.F., B. Bister, D. Bischoff, G. Nicholson, G. Jung, R.D. Süssmuth and F. Jüttner, Oscillapeptin J, a new grazer toxin of the freshwater cyanobacterium *Planktothrix rubescens*. Journal of Natural Products, 2003. **66**: 431-434.

16. Blom, J.F., J.A. Robinson and F. Jüttner, High grazer toxicity of [D-Asp^3, Dhb^7]microcystin-RR of *Planktothrix rubescens* as compared to different microcystins. Toxicon, 2001. **39**: 1923-1932.

Submitted:

17. Blom, J.F., Y. Bethuel, F. Jüttner and K. Gademann, Allelopathic activity of the iron chelator anachelin - a molecular hybrid with a dual mode of action*. Submitted*.

B) Patent

18. Blom, J.F. and K. Gademann. 2005. Antifouling Agents*.* # EP1783128 A1

C) Book Chapter

19. Blom, J.F., S.J. Hoeger and F. Jüttner (2010) Characterization of bioactive cyclic oligopeptides of freshwater cyanobacteria (microcystins, cyanopeptolins, cyclamides). *In* S.N. Bagchi, D. Kleiner, and P. Mohanty (Eds), Protocols on Algal and Cyanobacterial Research, Narosa Publishing: New Delhi, India, pp.53-70

List of Presentations

Dr. Judith Blom

***denotes invited lectures**

'Rapid evolutionary adaptation of a Freshwater bacterium to intense grazing pressure'
SAME (Symposium on Aquatic Microbial Ecology) meeting, Rostock, Germany; September 2011 *(Poster presentation)*

'Scent of danger: Conspecific chemical cues induce aggregate formation in a freshwater bacterial strain'
SIL (Societas Internationalis Limnologicae), Cape Town, South Africa, August 2010 *(Oral presentation)*

***** Plenary Talk: 'Experimental design and microbial species interactions'
International Student Course, SItE*MICRO*, Rome, Italy; May 2010 *(Oral presentation)*

'Aggregate formation in a freshwater bacterial strain induced by conspecific chemical cues'
SAME (Symposium on Aquatic Microbial Ecology) meeting, Piran, Slovenia; August 2009 *(Oral presentation)*

***** 'Microbial Chemical Interactions'
Aquatic Botany Group, Limnology, University of Konstanz, Germany; May 2009 *(Oral presentation)*

'Chemical interactions between *Sphingomonas* sp. and *Poterioochromonas* sp.'
Issues and Perspectives in Aquatic Microbiology and Microbial Ecology, Wierzba, Poland; May 2008 *(Oral presentation)*

***** 'Chemical Interactions between free-living pro- and eukaryotic cells'
Chemical Synthesis Laboratory, EPFL, Lausanne, Switzerland; February 2008 *(Oral presentation)*

***** 'Chemical defence of cyanobacteria'
Institute of Limnology, Austrian Academy of Sciences, Mondsee, Austria; November 2006 *(Oral presentation)*

'Oscillapeptin J, a new bioactive compound of *Planktothrix rubescens* and its impact on different organisms'
IVth International Conference on Toxic Cyanobacteria, Bergen, Norway; June 2004 *(Oral presentation)*

***** 'Secondary metabolites of cyanobacteria'
Institute of Limnology, University of Konstanz, Germany; May 2004 *(Oral presentation)*

'Oscillapeptin J, kein Microcystin und trotzdem toxisch'
Jahrestagung der Deutschen Gesellschaft für Limnologie (DGL), Cologne, Germany; September/October 2003 *(Oral presentation)*

'Wie toxisch ist der Zürichsee?"
Centre for Xenobiotic and Environmental Risk Research (XERR), Kilchberg, Switzerland, 2003 *(Oral presentation)*

***** '*Planktothrix rubescens*, its toxicity and function in Lake Zürich"
Institute for Environmental Toxicology, University of Konstanz, Germany; May 2002 *(Oral presentation)*

'Ungewöhnlich hohe Grazertoxizität des Hauptmicrocystins [D-Asp^3,(*E*)-Dhb^7]microcystin-RR von *Planktothrix rubescens'*
Jahrestagung der Deutschen Gesellschaft für Limnologie (DGL), Kiel, Germany; September/October 2001 *(Oral presentation)*

'Grazer toxicity analysis of [D-Asp^3,(*E*)-Dhb^7]microcystin-RR from *Planktothrix rubescens'*
IX. Congress of Toxicology, Brisbane, Australia; August 2001 *(Poster presentation)*

'Wie toxisch ist der Zürichsee?'
Jahrestagung der Schweizerischen Gesellschaft für Hydrologie und Limnologie (SGHL) Winterthur, Schweiz; October 2000 *(Oral presentation)*

'Toxicity of the major *Planktothrix rubescens* microcystin and other microcystin derivates against invertebrate grazers'
Aquatic Sciences Meeting of the American Society of Limnology and Oceanography (ASLO)" in Copenhagen, Denmark; June 2000 *(Poster presentation)*

'In vivo measurement of intrinsic NADH fluorescence in copepods (*Temora longicornis*) and plaice embryos (*Pleuronectes platessa*)'
International Symposium on Animal Physiology, Berlin-Bogensee, Germany; June 1998 *(Poster presentation)*